

MICROBIAL GROWTH ON ANTHROPOGENIC COMPOUNDS BY  
REDUCTIVE DEHALOGENATION OR OXIDATION

A Dissertation

Presented to the Faculty of the Graduate School  
of Cornell University

In Partial Fulfillment of the Requirements for the Degree of  
Doctor of Philosophy

by

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January 2013

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# MICROBIAL GROWTH ON ANTHROPOGENIC COMPOUNDS BY REDUCTIVE DEHALOGENATION OR OXIDATION

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Cornell University 2013

The improper disposal of chlorinated organic compounds is a concern because of their toxicity and persistent nature. *Dehalococcoides mccartyi* strain 195 can reductively dehalogenate some of these compounds and, in the case of tetrachloroethene (PCE), detoxify it by producing ethene. Its genome contains a putative prophage, which could inhibit its ability to grow to high densities and cause phage mediated cell death. This phage was evaluated by genomic comparisons and electron microscopy. Cellular stress induced phage expression and stopped dechlorination.

*Dehalococcoides* can partially dechlorinate polychlorinated benzenes but will not use dichlorobenzene. Three strains of *Dehalobacter* that are able to produce monochlorobenzene from dichlorobenzene (DCB) were sequenced and assembled. Analysis of their genomes shows metabolic specialization for growth by reductive dehalogenation. Their genomes were larger than *Dehalococcoides* spp. genomes but smaller than the nearest sequenced phylogenetic relative, further indicating specialization.

The process of reductive dechlorination does not completely explain the flux of

chlorinated ethenes at contaminated sites. To investigate oxidation as an alternative metabolism, groundwater and sediment microcosms were amended with various electron acceptors and either vinyl chloride (VC) or ethene as electron donors. Determination of oxidation was monitored by gas chromatography for the loss of VC and ethene overtime without the production of ethene, ethane or methane.

From the groundwater microcosms, a *Mycobacterium* was isolated that could oxidize VC at microaerobic levels, perhaps partially explaining the observed lack of mass balance. Growth was monitored visually and increased with the decrease in VC concentration.

From the sediment microcosms, an enrichment culture was developed, with one dominant organism related to *Desulfovirga adipica* that was able to oxidize ethene coupled to sulfate reduction. Growth and metabolism in this enrichment culture was observed by quantitative PCR and the production of sulfide.



## BIOGRAPHICAL SKETCH

Heather Elizabeth Fullerton was born on November 4, 1982 in Kirkland Washington to Noel Fullerton. She learned a love and appreciation for the natural world from stepfather, Neal Underland. Both Neal and Noel inspired her to ask questions about the world around her and to think critically about it. Her curiosity about the natural world, led her to take many science electives while attending Juanita High School. One such class was Microbiology where she fell in love with the microscopic world.

To further her education, she attended University of Washington for four years and graduated in 2005 with a Bachelor's of Science in Microbiology. To gain laboratory experience while attending university she worked in a variety of different laboratories focused on topics different from her scientific interest. These laboratories focused on a variety of biological questions, such as human thrombocytopenia and mouse muscle regeneration. However, she never faltered in her love for the microscopic world.

After spending a year as a laboratory technician working on cell signaling in the developing mouse muscle, she started her graduate career in the Department of Microbiology at Cornell University in August of 2006. Interested specifically in the diversity of microbial respiration and microbial life styles, she started working with Dr. Stephen Zinder on metabolism of anthropogenic compounds. Upon completion of her degree, Heather will work on marine microbial ecology and geomicrobiology of hydrothermal vent systems with Dr. Craig Moyer at Western Washington University.

This work is dedicated to my family for their unwavering  
support during my entire educational pursuit

## ACKNOWLEDGMENTS

There are many individuals who I would like to express my eternal gratitude and appreciation for their support and help in my educational pursuit at Cornell University. For being a wonderful and patient advisor, Dr. Stephen Zinder for without his support and insights, none of this would have been possible. His breadth of knowledge and enthusiasm of all things microbial is astonishing and inspiring.

I would like to express my thanks to my family for keeping me grounded and providing me with support throughout this time even though I was so far from home. My parents Neal and Noel, have always been willing to listen to me and advise me, and always believed I could and would do my best at anything I set my mind to. They made me realize that growing up does not mean growing apart and you're never too old to be comforted by a hug from your parents. And in growing up, I've learned to listen to my older, wiser, sister Sara who is continually right even if I don't listen to her sage words.

A special thanks to my friends Heather Dooley and Loren Donner who have been supportive and a shining light in rough times. They could make me laugh even on the darkest day, and have listened to my troubles and worries without judgment and would always remind me of who I actually am.

I would also like to thank the many friends and colleagues that have provided me with scientific and personal insight throughout the years. To the lunch crew, Julie Brown, Armanda Roco, Tony Kingston, Greg Smaldone, Letal Salzberg, and Erin Eggleston, for providing me with a much needed daily break. To my lab mates, past

and present, Jenny Nelson and Jen Fung, for making the work day fun and providing valuable scientific advice. This department would have failed to function had it not been for Shirley Cramer, Cathy Shappell, and Patti Butler, and to Barb Eagleston for her aid with the microscopes.

The brewing conspiracy, Ben and Gretchen Heavner, Cresten Mansfeldt, Cloe Giddings, Annie Rowe, Mike Booth, Sarah Short, Matt Rendina, Madeline Galac, Rick Pampuro, and Punita Juneja, who always made sure I had fresh homebrew in the fridge. For without them I'd never had learned to brew beer, made pizza dough, cross-country skied, snow shoed, camped in a yurt in winter, played Carcassonne and other board games, swam across Cayuga lake, driven to DC for a rally, or have danced the night away until the cops are called. Without these people adding excitement and adventure, getting a PhD would have been a lot less fun.

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## CHAPTER ONE

### OVERVIEW: GROWTH ON CHLORINATED ANTHROPOGENIC COMPOUNDS

#### **Context**

Anthropogenic compounds are a diverse class of chemicals, many of which can be metabolized by humans and microbes. Some of these chemicals are classified as known or potential carcinogens and have been released into the environment. The Environmental Protection Agency (EPA) has designated a large number of these compounds as priority pollutants. One such family of compounds is chlorinated organics, such as tetrachloroethene (PCE) and dichlorobenzene (DCB). These compounds are present on the Agency for Toxic Substances and Disease Registry (ATSDR) toxic substances list, where the ranking of each compound is calculated based on potential for human exposure, frequency of occurrence at contaminated sites, and toxicity. Many of the chlorinated organics are particularly tricky to mitigate since they are denser than water and form dense nonaqueous phase liquids (DNAPLs) that migrate to anoxic zones within a contaminant plume. Chloroorganic compounds range in rank (Table 1.1), from the most toxic organic compound (VC, ranked 4<sup>th</sup>) to the least (1,2,3,7,8,9-hexachlorodibenzofuran, ranked 275<sup>th</sup>).

ATSDR 2011 Substance Priority List	
Compound	Rank
Vinyl Chloride	4
Polychlorinated Biphenyls	5
Benzene	6
Trichloroethylene	16
Tetrachloroethylene	33
Pentachlorobenzene	77
1,1-Dichloroethene	81
Hexachlorobenzene	93
Monochlorobenzene	117
1,2,3-Trichlorobenzene	139
1,4-Dichlorobenzene	159
Heptachlorodibenzofuran	166
1,2 <i>trans</i> -Dichloroethene	177
1,2-Dichlorobenzene	175
1,2,4-Trichlorobenzene	199
1,3-Dichlorobenzene	204
1,2 <i>cis</i> -Dichloroethene	270
1,2,3,7,8,9-hexachlorodibenzofuran	275

Table 1.1: Priority ranking of selected chlorinated compounds and benzene (23).

Chlorinated ethenes are toxic, anthropogenic compounds that have many industrial and commercial uses. Due to improper disposal, leakage of storage vessels, and the persistent nature of these compounds, they have polluted aquifers and landfills and remain one of the most common groundwater pollutants. Many of the characteristics that make these chemicals appealing to use, contribute to long-lasting contamination. The Safe Drinking Water Act dictates that many of these compounds have a maximum contaminant level of 5 µg/liter, with VC having a maximum contaminant level of 2 µg/liter.

Traditional remediation methods, such as pump and treat or monitored natural attenuation of PCE and trichloroethene (TCE) are often expensive. Natural attenuation

results often vary and result in higher toxicity levels of groundwater due to build up of dichloroethene (DCE) or vinyl chloride (VC) formed via reductive dechlorination.

Bioremediation has been pursued as a viable option for removal of chlorinated ethenes but this process has disadvantages. Under aerobic conditions PCE and TCE fail to be efficiently metabolized whereas DCE and VC can be biodegraded. Most contaminant plumes are anaerobic (21). PCE and TCE can be metabolized efficiently in highly reduced anaerobic environments, wherein chlorines are sequentially replaced with hydrogen in a process termed reductive dehalogenation as illustrated in Figure 1.1.

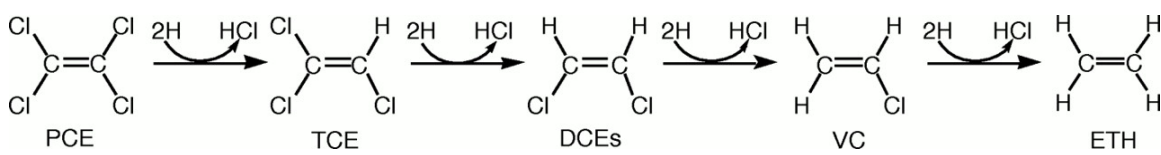


Figure 1.1: Reductive dehalogenation of PCE to Eth as carried out by *Dehalococcoides mccartyi* strain 195

Reductive dehalogenation is thermodynamically favorable for both chlorobenzenes and chloroethenes (26, 32). For example, the  $\Delta G^{\circ}$  for reductive dechlorination of 1,2 dichlorobenzene to monochlorobenzene is -153.2 kJ/reaction, for PCE to TCE the  $\Delta G^{\circ}$  is -173.8 kJ/mole and for reduction of VC to ethene the  $\Delta G^{\circ}$  is -149.9 kJ/mole, making these reactions thermodynamically favorable (26). Total reductive dechlorination of polychlorinated benzenes or ethenes results in either benzene (24, 55, 58) or ethene (60), which are highly toxic or nontoxic respectively.

In the case of chloroethenes, tracking and monitoring the production of ethene can be inconclusive (6, 54). Poor mass balance may show evidence of ethene metabolism rather than incomplete dechlorination (62).



Phylum	Microorganism	Isolated for growth on	e- donor	Enzyme Class
<i>Chloroflexi</i>	<i>Dehalococcoides mccartyi</i> 195	Perchloroethene	H <sub>2</sub>	Reductive Dehalogenase
	<i>Dehalococcoides mccartyi</i> CBDB1	1,2,3-Trichlorobenzene	H <sub>2</sub>	Reductive Dehalogenase
	<i>Dehalococcoides mccartyi</i> BAV1	Dichloroethene	H <sub>2</sub>	Reductive Dehalogenase
	<i>Dehalococcoides mccartyi</i> VS	Dichloroethene	H <sub>2</sub>	Reductive Dehalogenase
<i>Firmicutes</i>	<i>Dehalobacter Restrictus</i>	PCE	Formate, H <sub>2</sub>	Reductive Dehalogenase
	<i>Desulfitobacterium hafiense</i> Y51	Perchloroethene	Formate, lactate, pyruvate	Reductive Dehalogenase
	<i>Desulfitobacterium hafiense</i> DCB-2	2,4,6-Trichlorophenol	Formate, lactate, pyruvate	Reductive Dehalogenase
<i>ε-Proteobacteria</i>	<i>Sulfurospirillum multivorans</i>	Perchloroethene	Formate, H <sub>2</sub>	Reductive Dehalogenase
<i>δ-Proteobacteria</i>	<i>Geobacter lovleyi</i> SZ	Perchloroethene	Acetate, Pyruvate, H <sub>2</sub>	Reductive Dehalogenase
	<i>Polaromonas</i> JS666	cDCE	Oxygen	Monooxygenase
<i>Actinobacteria</i>	<i>Mycobacterium</i> JS60	VC	Oxygen	Monooxygenase
	<i>Nocardioideis</i> JS614	VC	Oxygen	Monooxygenase

Table 1.2: A subset of organisms capable of metabolism of chlorinated compounds and the enzyme class responsible for mediating this metabolism

This chapter describes a few of the organisms responsible (listed in Table 1.2) for metabolism of a few anthropogenic compounds by two mechanisms:

1. Reductive Dehalogenation, the use of chloroorganic compounds as electron acceptor
2. Oxidation, the use of a chlorinated compound as the electron donor and the sole source of carbon and energy

Understanding of the organisms responsible for mediating these two processes will provide insights into natural attenuation and potential bioremediation.

## Reductive Dehalogenating Organisms and Processes

*Dehalococcoides mccartyi* spp. are known to use H<sub>2</sub> as the only electron donor; electron acceptors are limited to halogenated compounds, and acetate is used as the only carbon source. Phylogenetic analysis by 16S rRNA gene sequences of these organisms

places *Dehalococcoides* within a deep branching subphylum of the *Chloroflexi* (also called green nonsulfur bacteria). The type strain of this species, *D. mccartyi* strain 195 (previously “*D. ethenogenes*”) is the only known organism that can completely dechlorinate PCE to non-toxic ethene (60).

Other strains of *Dehalococcoides* are unable to fully metabolize PCE to ethene (28). *D. mccartyi* strains 195 and FL2 cometabolically dechlorinate VC to ETH leading to considerable VC accumulation, whereas strains BAV1, VS, and GT metabolically dechlorinate VC to ETH (41, 74). Strain CBDB1 can use PCE and TCE, with *trans*-DCE as the primary end product. Strains GT and VS are unable to metabolize PCE, whereas strain BAV1 can cometabolize PCE and TCE to produce ethene, and strain FL2 can cometabolize PCE and VC to produce ethene (28). *Dehalococcoides* spp. have a range of substrate utilization patterns.

Various halogenated compounds have been shown to be dehalogenated by *Dehalococcoides* spp. including polychlorinated biphenyls (PCBs), penta-brominated diphenyl ethers (PBDE), polychlorinated dibenzo- p-dioxins, and dibenzofurans (PCDD/F), and polychlorinated benzenes (5, 27, 45, 46, 53), most of which are present on the ATSDR list of priority compounds (Table 1.1). *D. mccartyi* strain 195 can also dehalogenate chlorobenzenes with doubly-flanked chlorines; however it cannot dehalogenate trichlorobenzenes or dichlorobenzenes. *Dehalobacter* strains have been identified with the ability to completely dehalogenate chlorobenzenes to benzene (63).

The growth of strain 195 in pure culture is slow with low biomass yields. The genome of strain 195 has been extensively analyzed and the presence of an apparent complete prophage identified. Prophages can have dire consequences on microbial

growth and could be responsible for low biomass yields of strain 195 in pure culture (4, 19, 30, 33). Chapter two presents data that shows this prophage to be active and inducible in strain 195.

The process of reductive dechlorination is not ubiquitous at contaminated chloroethene sites and is dependent on the specific organisms present (43). *Dehalobacter* spp., *D. hafniense*, *Geobacter lovleyi*, *Sulfurospirillum* (*Dehalospirillum*) *multivorans* are only able to reduce PCE to *cis*-DCE via TCE (2, 65, 66).

*Dehalobacter* spp. can utilize different dichlorobenzene isomers, dichloromethane, chloroform, tetrachlorophthalide, and trichloroethane (37-39, 48, 64, 78). The analyses of three genomes of DCB dehalogenating DHBs are discussed in chapter three.

*Dehalobacter* (DHB) spp. are phylogenetically distinct from *Dehalococcoides*, belonging to the *Firmicutes* (44). *Dehalobacter restrictus* strain PER-K23, the type strain, was isolated with PCE as the electron acceptor, hydrogen as electron donor, and acetate as carbon source. Though this is a similar metabolic lifestyle as *Dehalococcoides*, *D. restrictus* can be grown in defined medium with the addition of two vitamins, (thiamine, cyanocobalamin) and three amino acids (arginine, histidine, threonine) whereas *D. mccartyi* can grow without supplemental amino acids (28, 44). DHBs are obligate anaerobes and are most closely related to *Desulfitobacterium hafniense*, another *Firmicutes* that is able to grow by reductive dehalogenation, respiration with sulfite or fumarate as the electron acceptor, or by fermentation (49, 67, 77).

A class of enzymes called reductive dehalogenases (rdh) is common to dechlorinating organisms. This enzyme class is characterized by the presence of a

corrinoid cofactor and two 4Fe-4S clusters (57). Each dechlorinating organism has at least one rdh and some have as many as 38, though the function of each rdh is unknown. Studies using *Dehalococcoides* are able to link some of the rdh genes to a specific function. This is done with a combination of protein gel assays, reverse genetics and quantitative PCR (1, 31, 61). In strain 195, PceA was identified as the protein responsible for dehalogenation of PCE to TCE, and TceA as responsible for dehalogenation of TCE to ethene although the final chlorine removal is cometabolic (31, 56). In strain 195, expression of the rdh genes are mediated by flanking regulatory proteins, the chloroorganic compounds present and the respiration rate (52, 72).

### **Oxidation of VC and Ethene**

Since not all dehalogenating organisms can metabolize chloroethenes to non-toxic ethene, VC accumulation can occur (43, 59). VC can be detected in anaerobic zones of chloroethene-contaminated sites, though sometimes it disappears without the detection of corresponding amounts of ethene, indicating that VC or ethene is being metabolized by an alternative pathway within the anaerobic zone (13, 43).

Production of  $^{14}\text{C}$ -acetate (8),  $^{14}\text{CO}_2$ , and  $^{14}\text{CH}_4$  (11, 14), from  $^{14}\text{C}$ -labeled VC has been observed in microcosms, but no transformations have been described for unlabeled VC at greater than tracer quantities. However, it remains unknown which microbe might be performing this metabolism (15). Previous studies have reported that VC mineralization could be coupled to reduction of Fe(III) or humic substances (9, 10, 18). Aerobically, VC oxidation has been observed in *Mycobacterium* spp., *Nocardioides* spp. (21, 76) and *Pseudomonas* spp. (76) among others.

It is still unclear in many cases whether VC oxidation detected in cultures and microcosms was truly anaerobic or due to contamination by trace amounts of oxygen. Chapter four describes anaerobic cultures that apparently oxidized non-tracer concentrations of VC. The best explanation for the behavior of the culture is infiltration of small amounts of oxygen sufficient to oxidize the VC in those cultures as demonstrated by culturing and molecular biological methods.

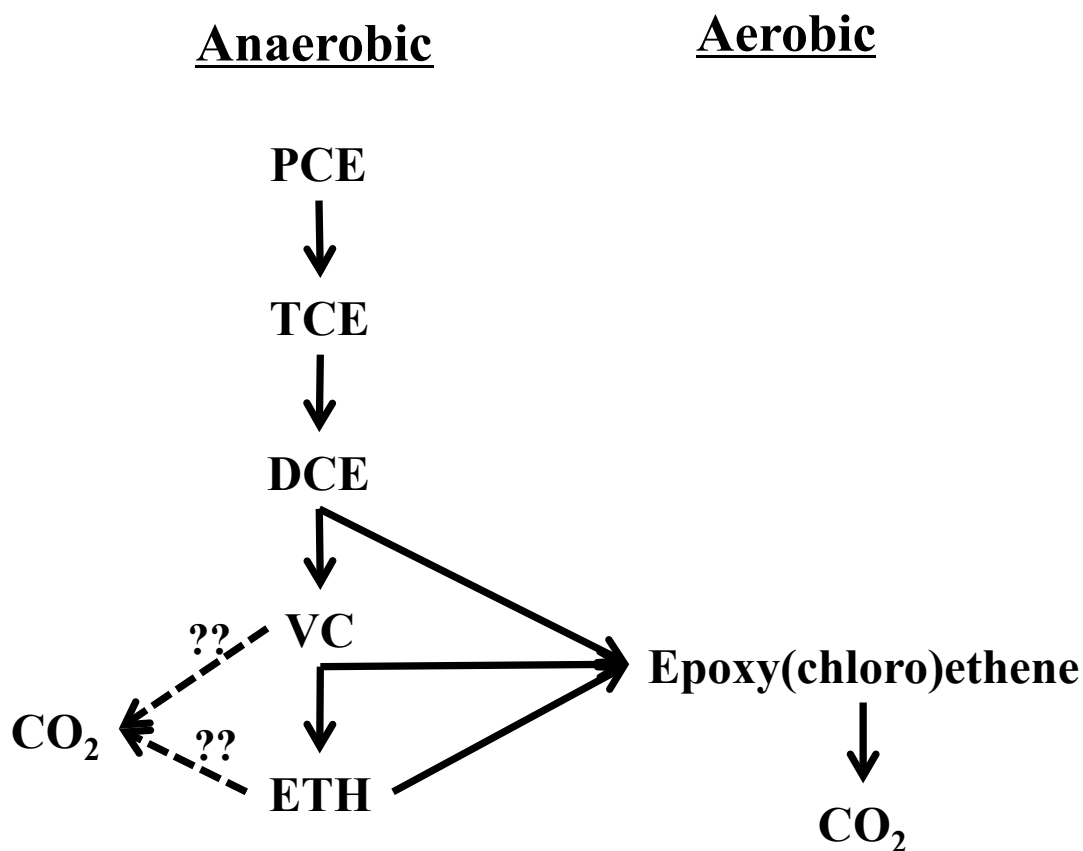


Figure 1.2: Fate of chloroethenes in groundwater systems. Dashed lines represent thermodynamically possible reactions, without an identified mechanism or organism.

The aerobic conversion of ethene, VC and DCE are known to be mediated by a specific class of enzymes, monooxygenases (22). These enzymes convert ethene, VC and DCE into their corresponding epoxides. These epoxides are then metabolized and in the

case of *Mycobacterium* JS60 grown on ethene to 2-hydroxyethyl-CoM (22, 47). This transformation can only happen in the presence of oxygen, which is the reactant. Oxygen levels at the edge of a contaminant plume could be below detection limit, yet the flux of oxygen could still be high enough to allow for aerobic growth (35).

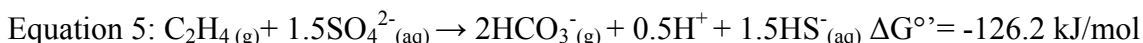
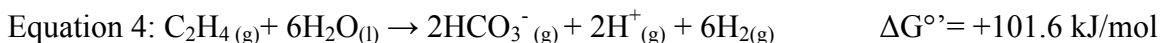
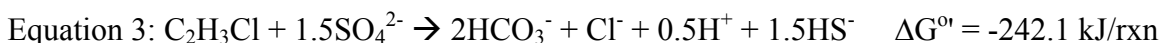
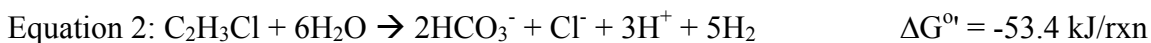
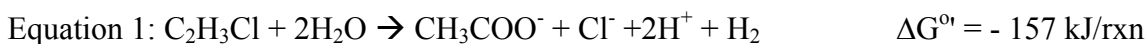
*Polaromonas* sp. strain JS666 is the only isolated bacterium capable of growth on cDCE as its sole carbon and energy source (20, 47). When grown on cDCE, this isolate could also metabolize *trans*-DCE, TCE, VC, 1,2-DCA and ethene. The metabolic mechanism for growth on cDCE by JS666 appears to be the same as for growth on VC and ethene, with formation of an epoxide catalyzed by a monooxygenase (47).

There are a few theories explaining the failure to detect ethene in anaerobic zones, despite the observed decrease in VC concentrations (7, 16, 17, 35):

1. Ethene produced from reductive dechlorination is rapidly oxidized to CO<sub>2</sub> anaerobically
2. VC is directly oxidized to CO<sub>2</sub> anaerobically
3. VC is oxidized to acetate which is further metabolized to CO<sub>2</sub> and CH<sub>4</sub>
4. Oxygen is continually introduced and consumed keeping it below detection limits, allowing for the aerobic oxidation of VC

The presence of an aerobic zone in plumes might not fully explain the lack of mass balance at contaminated sites (54, 59). The conversion of VC to either acetate (Equation 1) or to CO<sub>2</sub> and H<sub>2</sub> (Equation 2) is energetically favorable under anaerobic conditions with a  $\Delta G^{\circ'}$  of -157 kJ/reaction and -53.4 kJ/reaction, respectively. Using a terminal electron acceptor such as sulfate increases the favorability of the reaction to -242.15 kJ/reaction (Equation 3) (75). The possibility that ethene is oxidized anaerobically

as soon as it is formed is also thermodynamically favorable under sulfate reducing conditions (Equation 5).



Ethene is a non-toxic, gaseous, phytohormone, and is been produced in large quantities for plastic manufacture (40, 51). The aerobic organisms that are able to metabolize ethene are common in soils and the natural accumulation of ethene from plants is minor. If the soils become water logged, anaerobic conditions will develop, which will prevent the metabolism of ethene (29, 34, 69).

Ethene has been considered recalcitrant under anaerobic condition and is inhibitory to methanogenesis (70, 71). Short chain hydrocarbons such as methane, ethane, propane, and butane are components of natural gas that can be metabolized aerobically and anaerobically (3, 42). The anaerobic metabolism of these hydrocarbons coupled to sulfate reduction, has been reported (50, 59). Anaerobic metabolism of short and long chain hydrocarbons has been linked to sulfate reducing organisms (36). The anaerobic oxidation of ethene has only been reported in trace amounts and the reduction to ethane has been sporadically reported (12, 25).

Since reductive dehalogenating organisms are obligate anaerobes, ethene production from the dechlorination would be in an anaerobic zone (73). A few studies

have reported the metabolism of ethene anaerobically, by reduction to ethane (Equation 6) or mineralization to  $\text{CO}_2$  (12, 25). As equation 5 shows, the oxidation of ethene is thermodynamically favorable when coupled to sulfate reduction. No organisms have been identified as responsible for the anaerobic mineralization of ethene. The development of enrichment cultures showing anaerobic oxidation of ethene coupled to sulfate reduction will be further discussed in chapter five.

Thus, the known microbially mediated degradation of hydrocarbons has not included the anaerobic degradation of ethene, whereas methane, ethane, acetylene and longer chain hydrocarbons are metabolized (68). Understanding the anaerobic fate of ethene is important because the production of ethene is often used in conjunction with the decrease in chloroethene concentration as evidence for bioremediation (54).

Investigations into the anaerobic metabolism of ethene will further our understanding of hydrocarbon metabolism and could reduce uncertainty about bioremediation at chloroethene contaminated sites.



## Objectives

The remediation of contaminated sites will benefit from the increased understanding the organisms that can metabolize chloroorganic compounds. While *Dehalococcoides* spp. are able to completely dechlorinate chloroethenes, this species alone cannot fully explain field site metabolite flux at field sites and lack of mass balance (13, 54, 62). *Dehalococcoides* genomic analysis revealed the presence of an apparent intact prophage (72). Studies on this prophage could help in understanding failure to remediate even though the organisms and conditions would seem to favor reductive dechlorination. This is the first objective, which is the focus of chapter two.

There are other organisms capable of reductive dehalogenation besides *Dehalococcoides*. One such organism is *Dehalobacter*, which can grow by reductive dechlorination of PCE to cDCE (44). Three strains of *Dehalobacter* have been characterized on their ability to dechlorinate polychlorinated benzenes (63). The second objective is to analyze the genomes of these metabolically specialized organisms. This objective is elaborated on in chapter three.

Because of incomplete dechlorination of PCE by organisms like *Dehalobacter* and *Dehalococcoides* strains, identification of organisms that are capable of oxidizing ethene or VC anaerobically will help to reduce uncertainty during remediation. This is the third objective and the focus of chapters four and five.

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CHAPTER TWO

THE LIFE CYCLE OF PROPHAGE  $\Phi$ D195 *DEHALOCOCCOIDES MCCARTYI*

STRAIN 195

**Introduction**

Chlorinated ethenes are some of the most common groundwater contaminants in the US and comprise some of the most toxic organic anthropogenic compounds. These compounds are remnants from dry cleaning operations and various industrial processes and manufacturing. Due to improper handling and discharge, these carcinogenic compounds have made it into groundwater. Through the process of reductive dechlorination, that is the replacement of chlorines with hydrogen, tetrachloroethene (PCE) can be complete detoxified to ethene. *Dehalococcoides mccartyi* strain 195 (previously "*D. ethenogenes*"), is the only organism known to couple growth with the reductive dechlorination of PCE to non-toxic ethene (19). The genome of this organism was sequenced and annotated and genome analysis has allowed for further understanding of its metabolism (27).

Sequencing of strain 195 identified nine potential integrated elements including one (IE, VII) that appeared to contain an intact prophage (27). This element was 55,258bp and contained open reading frames (ORFs) DET1066-1118, and it was noted that there was extensive synteny with *Bacillus cereus* 10987 prophage. Johnson *et.al.* (16) found that ORFs 1066-1104 from this region had higher transcript levels in gene arrays as the culture reached stationary phase, reminiscent of prophage induction.

Recently, metagenomic studies identified another prophage of *Dehalococcoides* from the KB-1 mixed culture (33), and showed that electron acceptor limiting conditions activated this prophage. Early studies of strain 195 grown in medium supplemented with extracts from anaerobic digesters noted phage presence by EM (18). These studies could not attribute the phage-like particles to strain 195 due to the presence of the extracts. Growth of strain 195 in medium lacking extracts allowed for the prophage of strain 195 to be further studied. Based on sequence analysis, this prophage is a predicted member of the *Siphoviridae* family, many of which are described as temperate phages and have been sequenced from viral particles or as part of a bacterial chromosome (5).

Phages play an important role in natural systems through predation and mediation of horizontal gene transfer. Prophages and other insertion elements are major contributors to variation between strains and can contribute important biological properties (7). Phage predation can have a strong impact on ecosystem function through nutrient cycling (30). In many ecosystems viral abundance is estimated to be much greater than total cell counts (10, 11). Aquatic environments have been the most studied for their phage and host, diversity and abundance.

Described here is the prophage named  $\Phi$ D195, the bacteriophage particles produced by strain 195, and some of its properties.

## **Methods**

### **Culture Conditions**

Strain 195 was grown in a defined medium (9). All culture media vials and tubes were prepared anaerobically in an anaerobic glove box (Coy Laboratory Products, Grass

Lake, MI). Basal medium (9) was dispensed into 120 ml serum vials or 27 ml Balch tubes with 50 ml or 10 ml of medium, respectively. Culture tubes and vials were sealed with Teflon-coated butyl stoppers. Culture tubes and vials were removed from the glove box and autoclaved. Headspace was flushed with sterile N<sub>2</sub> gas and the medium supplemented with sterile, anaerobic 5 mM sodium acetate, 0.2 mM L-Cysteine, 0.2 mM sodium sulfide, 1.0 mM sodium bicarbonate, 2 mM MOPS pH 7.4, 1 mM Ti(III)NTA and vitamin solution containing 0.05mg vitamin B<sub>12</sub> per liter (19, 21) and inoculated at 2% (vol/vol). Hydrogen gas was added to 0.3atm and neat PCE was added as previously described (19). Cultures were incubated at 35°C, shaking and inverted in the dark. Reductive dechlorination of PCE and production of daughter products were monitored as previously described (20), using a Perkin-Elmer 8500 gas chromatograph with flame ionization detector.

### **Microscopy**

SYBR Green I staining was done as described by Patel *et. al.* (2007). Samples were fixed in a phosphate-buffered saline (PBS) solution containing 2% (vol/vol) final concentrations formaldehyde for a minimum of 10 minutes. Fixed samples were then filtered onto a 0.02µm Anodisc membrane filters (Whatman, Maidstone, Kent, UK). Dried filters were then placed in a SYBR Green I solution (Invitrogen, Grand Island, NY) for 15 minutes and dried again before mounting on microscope slide with anti-fade reagent (1:1 PBS/glycerol, 0.1% p-phenylenediamine). Slides were visualized and enumerated on an Olympus BX-51 epifluorescence microscope.

For electron microscopy 0.2ml of cells and medium were anaerobically removed from a culture that had consumed at least 2mmol/l PCE and applied to a formvar grid

(300 mesh, EMS, Hatfield, PA). This was mixed with an equal volume of 2% uranyl acetate on the grid. The stain and culture were dried under a stream of N<sub>2</sub>. Grids were briefly rinsed with MilliQ water (Millipore, Billerica, MA) and dried before observation. Cells were imaged on a Philips electron microscope equipped with a MicroFire digital camera and Optronics software.

### **Nucleic acid extraction**

DNA from cells and phage particles were not separated before DNA extraction. Culture medium was added to SDS to a 10% final concentration. This was then heated to 95°C for 10 minutes. DNA was isolated using the phenol/chloroform method as described by Fuhrman *et. al.* (1988)(12).

Purified phage DNA was isolated by centrifugation at 13,000 rpm for 30 minutes at 4°C to remove cells. Supernant was transferred to centrifuge tubes for PEG 8000 precipitation overnight at 4°C as described by Thurber *et. al* (32). Precipitated viral particles were concentrated by centrifugation at 13,000 rpm at 4°C. Most of the supernatant was removed and the pellet was resuspended in the remaining 100µl of supernatant. SDS was added to a 10% final concentration and samples were incubated at 95°C for 10 minutes. After heat denaturation, 1 ml of 100% ethanol and 150 µl of 10.5 M NH<sub>4</sub>Ac were added and the mixture was incubated overnight at -20°C for precipitation. Then samples were centrifuged at 13,000 rpm at 4°C and the supernatant removed. The DNA pellet was resuspended in 200 µl of virus-free dH<sub>2</sub>O. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added after resuspension. This mixture was centrifuged for 2 minutes at 13,300 rpm to separate out the aqueous layer. After centrifugation, the bottom layer was removed and an equal volume of

chloroform/isoamyl alcohol (10:1) was added, and the mixture was centrifuged again for 2 minutes at 13,300 rpm. Bottom layer was removed, and the DNA in the remaining sample, was precipitated as above. The resulting DNA pellet was washed with 70% EtOH and the samples were air dried to evaporate the remaining ethanol and the DNA was resuspended in 50µl dH<sub>2</sub>O.

### **Prophage induction**

Cultures of strain 195 were grown as described above. Vials were split into sterile anaerobic tubes after culture had consumed approximately 3 µl of PCE without a starvation period. Mitomycin C inductions were performed by adding mitomycin C (Sigma, St. Louis, MO) to a final concentration of 0.8µg/ml half of split culture. Hydrogen and PCE was added to all culture tubes then tubes were incubated in the dark at 35°C for 24 hours inverted and shaking. Dechlorination was monitored as described above and previously (20).

### **Phylogenetic tree**

Amino acid sequences were aligned first using ClustalW (31). The resulting alignment was then used to create a phylogenetic tree using the MrBayes within Geneious (17). *Granulicatella adiacens* ATCC49175 was used as an outgroup using poisson substitution model and gamma rate variation (15). Chain length set at 1,100,000 with a subsampling frequency of 200. Priors were set with an unconstrained branch length (15).

Gene Target	Amplicon Size (bp)	Primer Name	Sequence (5' - 3')
RNA Methyltransferase	185	DET 1065 F	TACCGCAATCATGCCCGTTT
		DET 1065 R	TTTATTGCTTGCGTGGCGGA
Hypothetical Protein	197	DET 1066 F	AACCACGAGTGCAAACGAGT
		DET 1066 R	TCTGGGACAATTGCCAGCAT
Recombinase	178	DET 1067 F	TACCAAGGATGAGAACGGCAACCT
		DET 1067 R	TCTTGTGATGGTGCTTGTGTGCC
Endolysin	171	DET 1070 F	AACGGCAAATACATCCGTGGCT
		DET 1070 R	ACCTTTCCTGCACAGCAGAAT
Hypothetical Protein	151	DET 1075 F	ATTGGCACCAATGGCGAACA
		DET 1075 R	ACATCAGCACTCGTCATGCT
Major Tail Protein	184	DET 1080 F	TAACAACAACGGCTATTCCGGCGA
		DET 1080 R	ACATGCAGTGACGGATCTTCCTCT
DNA Packing protein	193	DET 1084 F	AGGACAGCCTGATTGAAAGCCTGA
		DET 1084 R	AAGCGTCATATTGAGCGCATGGTG
DNA-Dependent Polymerase	153	DET 1100 F	AACTGACCACGGCCATGAAGGATA
		DET 1100 R	TGAAGTTCAGAGGGAGCGGTCTTT
Hypothetical Protein	182	DET 1104 F	TTTCATCTACGTTTCGAGCACC
		DET 1104 R	TGCAGCGTGATATTTCGAGT
Hypothetical Protein	229	DET 1105 F	ACCATTGATGATCCCAAGCCCA
		DET 1105 R	TTCTTTGGCAGCGTCGGAATGT
Septum formation MaF Protein	174	DET 1120 F	ATGCCGAAACCAAAGCCAGAGA
		DET 1120 R	TGCCGCTTATTACCGTATGGCT

Table 2.1: Primers used and developed for this study

### End point PCR and quantitative PCR

End Point PCR amplifications were performed with primer sets listed in table 2.1. Previously published primers targeting 16S rRNA gene, DHC385F and DHC695R (10), and *tceA*, *tceA*-500f and *tceA*-795r (13), were used to assess the *D. mccartyi* strain 195 genomic DNA content. The PCR reaction mixture contained 1.25U 5PRIME Taq polymerase, 1X 5PRIME Taq buffer, 0.3µM, 200µM dNTPs, and 2.5µl of extracted DNA in a total volume of 35µl. All PCRs were performed with an initial two minute 95°C step followed by 30 cycles of 95 °C for 1 minute, 50 °C for 1.5 minute, 72 °C for 1 minute followed by a 10 minute hold at 72 °C and a held at 4 °C until analysis. PCR amplicons were analyzed on a 1.25% TBE gel stained with ethidium bromide.

Quantative PCR amplifications were performed in triplicate on a BioRad MyiQ Cycler. Individual reactions contained iQ SYBR Green Super Mix (BioRad, Hercules,

CA), and primers at 200 nM. Previously published primers, DHC385F and DHC695R, targeting the 16S rRNA gene were used to quantify cell number (14). Primers were designed to target the prophage genes, not binding any other sequences in the genome of Strain 195. Primer sequences are listed in Table 2.1. Quantative PCR amplifications were carried out with the following parameters: 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Melting-curve analyses were used to screen for primer dimers. DNA target amplifications were compared to DNA standards obtained by serial dilution of genomic DNA.

## Results and discussion

### Genomic analysis

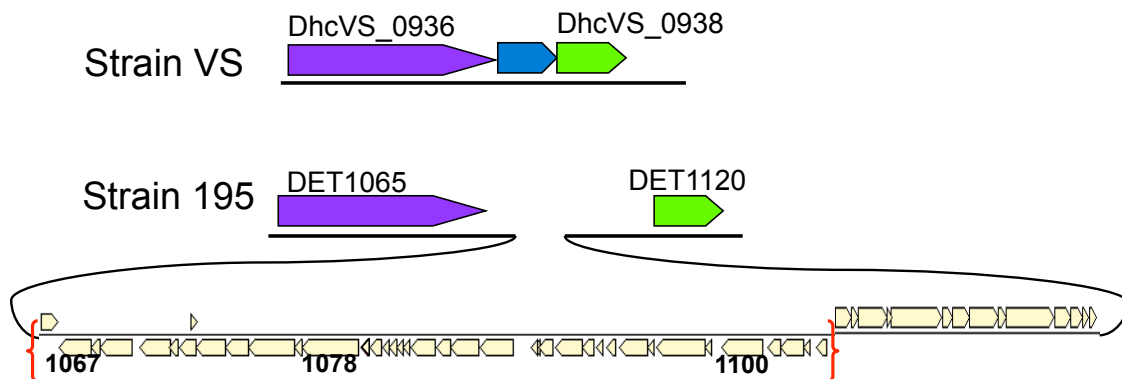


Figure 2.1: Genomic comparison between sequenced strains of *Dehalococcoides mccartyi* showing the genomic architecture of the viral integration site. In strain 195, the carbonic anhydrase (blue) is absent however; the RNA methyltransferase (purple) and maf-like (green) protein are conserved.

Comparisons between the genome of strain 195 and the other strains of *Dehalococcoides* with sequenced genome show that only this strain contains IE VII (27). In strain VS, for example, shows that ORF DhcVS0937 is replace by IE VII plus DET1065. DhcVS\_0937 is annotated as a hypothetical protein, although according to the

IMG annotation (jgi.img.doe.gov) resembles a carbonic anhydrase, and is not present in Strain 195, while the two flanking ORFs are present in Strain 195, as well as corresponding upstream and downstream regions.

Gene	Present in Phage DNA
DET1065	-
DET1066	+
DET1067	+
DET1070	+
DET1075	+
DET1080	+
DET1084	+
DET1100	+
DET1104	+
DET1105	-
DET1120	-
16S rRNA	-
tceA (DET0079)	-

Table 2.2: End-point PCR analysis of genes from annotated prophage and genome of strain 195. PCR products were compared to amplification from strain 195 genomic DNA and examined on a 1% agarose/TBE gel.

Using endpoint PCR to identify Strain 195 genes associated with purified viral particles (Table 2.2) found that all ORFs tested from 1066-1104 were present in the particles, whereas flanking genes, DET1065 and DET1105 were not found, nor were any other genes tested from Strain 195. ORFs DET1067-1104 are all transcribed in the same direction and many of them are annotated to encode phage functions (Table 2.3). The predicted element encoded by ORFs 1105-1118 are transcribed divergently from the prophage genes, and includes ORFs predicted to encode a Type III restriction/modification system, and is likely to represent a distinct genetic element from the predicted prophage. ORFs 1066-1104 occupy 35.4Kb of the genome, now called  $\Phi$ D195 (27).



Seshadri *et. al.* (27) noted extensive synteny with a prophage of *B. cereus*, an aerobic member of the *Firmicutes*, and shown in Figure 2.2 is an extension of this early observation, showing that the closest relatives are anaerobic *Firmicutes*. Many of the predicted gene products of Strain 195 prophage show >80% amino acid identity with genes from this phylum (Table 2.3) and one ORF 1088, predicted to encode a phage terminase, shows 97% identity. The top hit for this ORF, and several other ORFs, is a prophage of *Lachnospiraceae* bacterium 8\_1\_57FAA (Figure 2.2), sequenced from an inflamed biopsy tissue from a 22-year-old female patient with Crohn's disease (1). The 16S rRNA gene from the draft genome is nearly identical with that for *Ruminococcus torques*, commonly detected in gastrointestinal microbiota.

Figure 2.2 shows extensive regions of synteny and homology between the two prophage genomes, as well as regions with low homology, most of which are occupied by genes predicted to encode hypothetical proteins. Among other differences are that  $\Phi$ D195 has both a holin and endolysin gene, whereas *Lachnospiraceae* 8\_1\_57FAA only contains an annotated holin.  $\Phi$ D195 seems to be missing structural proteins, a tail component and a minor structural protein in comparison to the prophage of *Lachnospiraceae* 8\_1\_57FAA.

Table 2.3 Genome organization of  $\Phi$ D195

Locus Tag	Size (AA)	Predicted Function	BLASTP Analysis			
			Most Significant database match	Accession #	%AA ID	Predicted Function
DET1066	275	Hypothetical Protein	hypothetical protein EUS_18770 Eubacterium siraeum 70/3	CBK96940.1	52%	
DET1067	518	Integrase	phage integrase family Site-specific recombinase Lachnospiraceae bacterium 8_1_57FAA	ZP_07959266.1	93%	DNA Modification, Recombination
DET1068	138	Site-Specific Recombinase	site-specific recombinase Lachnospiraceae bacterium 8_1_57FAA	ZP_07959267.1	76%	DNA Modification, Recombination
DET1069	519	Site-Specific Recombinase	site-specific recombinase Megasphaera elsdenii DSM 20460	YP_004767060.1	88%	DNA Modification, Recombination
DET1070	491	Endolysin	lysozyme M1 Clostridium sp. SY8519	YP_004707784.1	60%	Lysis
DET1071	141	Holin	Lachnospiraceae bacterium 8_1_57FAA	ZP_0795927.1	88%	Lysis
DET1072	268	Hypothetical Protein	peptidase U32 Clostridium papyrosolvens DSM 2782	ZP_08193007.1	22%	Lysis
DET1073	104	Hypothetical Protein	3-oxoacyl-synthase Methylophaga aminisulfidivorans MP	ZP_08536537.1	32%	Fatty Acid Biosynthesis
DET1074	472	Hypothetical Protein	hypothetical protein BACPEC_01322 Bacteroides pectinophilus ATCC 43243	ZP_03462261.1	54%	
DET1075	369	Hypothetical Protein	hypothetical protein BACPEC_01321 Bacteroides pectinophilus ATCC 43243	ZP_03462260.1	57%	
DET1076	756	Hypothetical Protein	hypothetical protein BACPEC_01320 Bacteroides pectinophilus ATCC 43243	ZP_3462249.1	54%	
DET1077	120	Hypothetical Protein	hypothetical protein BACPEC_01319 Bacteroides pectinophilus ATCC 43243	ZP_03462258.1	62%	
DET1078	907	Tail Tape Measure Protein	TP901 family Tail tape measure protein Lachnospiraceae bacterium 8_1_57FAA	ZP_07959277.1	80%	Viral Particle Structure
DET1079	125	Hypothetical Protein	hypothetical protein HMPREF1026_01222 Lachnospiraceae bacterium	ZP_07959279.1	92%	Viral Particle Structure
DET1080	202	phi13 family major tail protein	phage major tail protein, phi13 family Ethanoligenens harbinense YUAN-3	YP_004092098.1	90%	Viral Particle Structure
DET1081	108	Hypothetical Protein	prophage pi2 protein 38 Lachnospiraceae bacterium 8_1_57FAA	ZP_07959281.1	85%	
DET1082	127	Hypothetical Protein	prophage pi2 protein 37 Lachnospiraceae bacterium 8_1_57FAA	ZP_07959282.1	83%	
DET1083	105	Phage Head-Tail Adaptor	phage head-tail adaptor Ethanoligenens harbinense YUAN-3	YP_004092095.1	83%	Viral Particle Structure
DET1084	95	DNA-Packaging Protein	phage protein Ethanoligenens harbinense YUAN-3	YP_004092094.1	63%	Viral Particle Structure
DET1085	405	Phage Major Capsid Protein, HK97 family	phage major capsid protein, HK97 family Ethanoligenens harbinense YUAN-3	YP_004092093.1	89%	Viral Particle Structure
DET1086	242	ATP-dependent Clp Protease, Proteolytic subunit ClpP	ATP-dependent Clp protease proteolytic subunit Lachnospiraceae bacterium 8_1_57FAA	ZP_07959286.1	95%	Viral Particle Structure
DET1087	444	HK97 Family Portal Protein	HK97 family Phage portal protein Lachnospiraceae bacterium 8_1_57FAA	YP_07959287.1	92%	Viral Particle Structure
DET1088	535	Terminase, Large Subunit	phage Terminase Lachnospiraceae bacterium 8_1_57FAA	ZP_07959288.1	97%	DNA Modification, Packaging
DET1089	66	Virulence-Related Protein	hypothetical protein HMPREF1026_01234 Lachnospiraceae bacterium 8_1_57FAA	ZP_07959291.1	90%	
DET1090	74	Hypothetical Protein	methyltransferase Lachnospiraceae bacterium 8_1_57FAA	ZP_07959292.1	89%	DNA Modification, Methylation
DET1091	231	Virulence-Related Protein	hypothetical protein HMPREF1026_01236 Lachnospiraceae bacterium 8_1_57FAA	ZP_07959293.1	82%	
DET1092	421	DNA-Methylase	DNA methylase Lachnospiraceae bacterium 8_1_57FAA	ZP_07959294.1	96%	DNA Modification, Methylation
DET1093	193	Hypothetical Protein	hypothetical protein HMPREF0990_01229 Lachnospiraceae bacterium 1_1_57FAA	ZP_08618835.1	92%	
DET1094	115	HNH Endonuclease Domain Protein	HNH endonuclease Ethanoligenens harbinense YUAN-3	YP_004092082.1	78%	DNA Modification, endonuclease
DET1095	142	Hypothetical Protein	Hypothetical protein HMPREF1026_01240 Lachnospiraceae bacterium 8_1_57FAA	ZP_07959297.1	92%	
DET1096	488	SNF2 Domain-containing Protein	SNF2 domain-containing protein Lachnospiraceae bacterium 8_1_57FAA	ZP_07959299.1	93%	DNA Modification, Structural
DET1097	95	Hypothetical Protein	VRR-NUC domain protein Clostridium sp. HGF2	ZP_07831616	75%	DNA Modification, endonuclease
DET1098	789	Virulence-Associated Protein E	putative P-loop ATPase Spirochaeta sp. Grapes	AEV30603.1	58%	
DET1099	109	Hypothetical Protein	hypothetical protein CLOSCI_03520 Clostridium scindens ATCC 35704	ZP_02433249.1	76%	
DET1100	662	DNA-Dependent DNA Polymerase, Family A	DNA-dependent DNA polymerase, family A Lachnospiraceae bacterium 8_1_57FAA	ZP_07959304.1	89%	DNA Modification, Polymerase
DET1101	190	Hypothetical Protein	phage protein Lachnospiraceae bacterium 8_1_57FAA	ZP_07959306.1	97%	
DET1102	379	Hypothetical Protein	phage protein Lachnospiraceae bacterium 8_1_57FAA	ZP_07959307.1	95%	
DET1103	108	Hypothetical Protein	DNA ligase Lachnospiraceae bacterium 8_1_57FAA	ZP_07959308.1	86%	DNA Modification, Structural
DET1104	173	Hypothetical Protein	hypothetical protein EUBELI_01534 Eubacterium eligens ATCC 27750	YP_002930973.1	80%	

Table 2.3: Locus tags and annotated function of genes encoded by  $\Phi$ D195 with their closest BLASTP database hit and percent identity

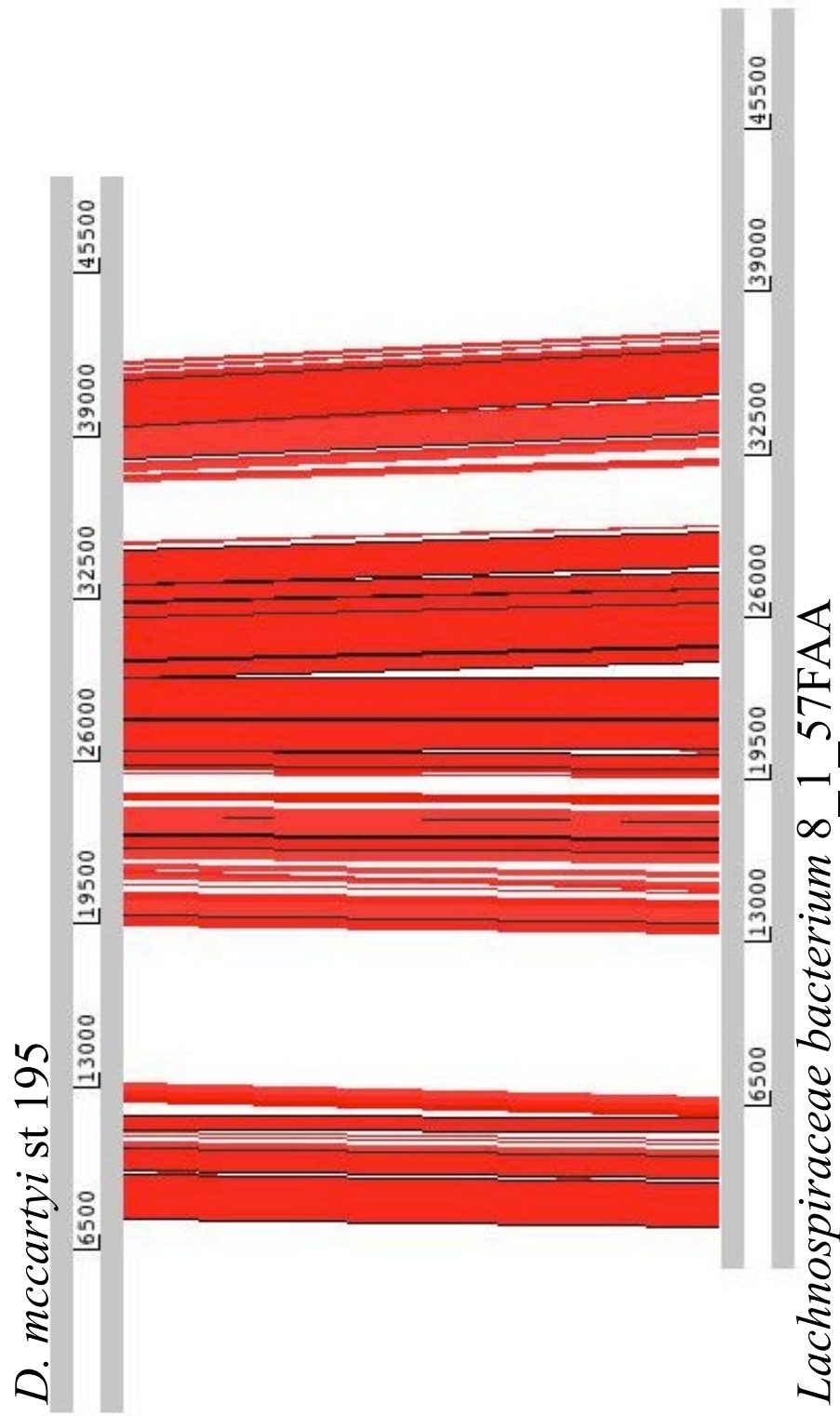


Figure 2.2: ACT comparisons at the nucleotide level between the prophage of *D. mccartyi* strain 195 and *L. bacterium 8\_1\_57\_FAA*. Comparison based on nucleotide sequences. The first gene to line up by this analysis is a site-specific recombinase gene (DET1067). These two prophages show a high degree of synteny.

The capsid protein of  $\Phi$ D195 has high identity with a well-studied group of capsid proteins, HK97. This type of capsid is very common among phages of *Enterbacteriaceae*. Most of the genes of  $\Phi$ D195 produce congruent phylogenetic trees. An example is the protein tree for DET1078, the predicted tail tape measure protein (Figure 2.3). *D. mccartyi* strain 195 is the only member of the *Chloroflexi* present in this gene tree, whereas the other organisms belong to the *Firmicutes*. This phylogenetic disparity indicates that  $\Phi$ D195 was recently acquired by strain 195 (24). There is no evidence of *Lachnospiraceae* present in the mixed community from which strain 195 was isolated (25). Strain 195 is the only strain of *Dehalococcoides* to have been cultured from a wastewater treatment plant, which are known to harbor great phage diversity (36) and contain large numbers of gastrointestinal bacteria like *Lachnospiraceae* bacterium 8\_1\_57FAA.

$\Phi$ D195 and the related phages in Figure 2.3 are members of the *Siphoviridae* virus family based on several gene sequences. This family of phages has a typical icosahedral nonenveloped head-tail structure, with non-contractile tail fibers, containing a linear, dsDNA genome. Many phages of the *Siphoviridae* family integrate into the host chromosome as prophages. Other phages of this family have been described as a vector for transmission of antibiotic resistance among *Streptococcus pyogenes* (4). All sequences represented in Figure 2.3 except for the *Listeria* phage, are from whole genome sequencing projects.

The genome of  $\Phi$ D195 carries typical genes for an integrative phage and is split into early and late genes (37). DET1067-1069 (Table 2.3) are annotated as integrases and recombinases, which most likely are responsible for phage genome integration into the

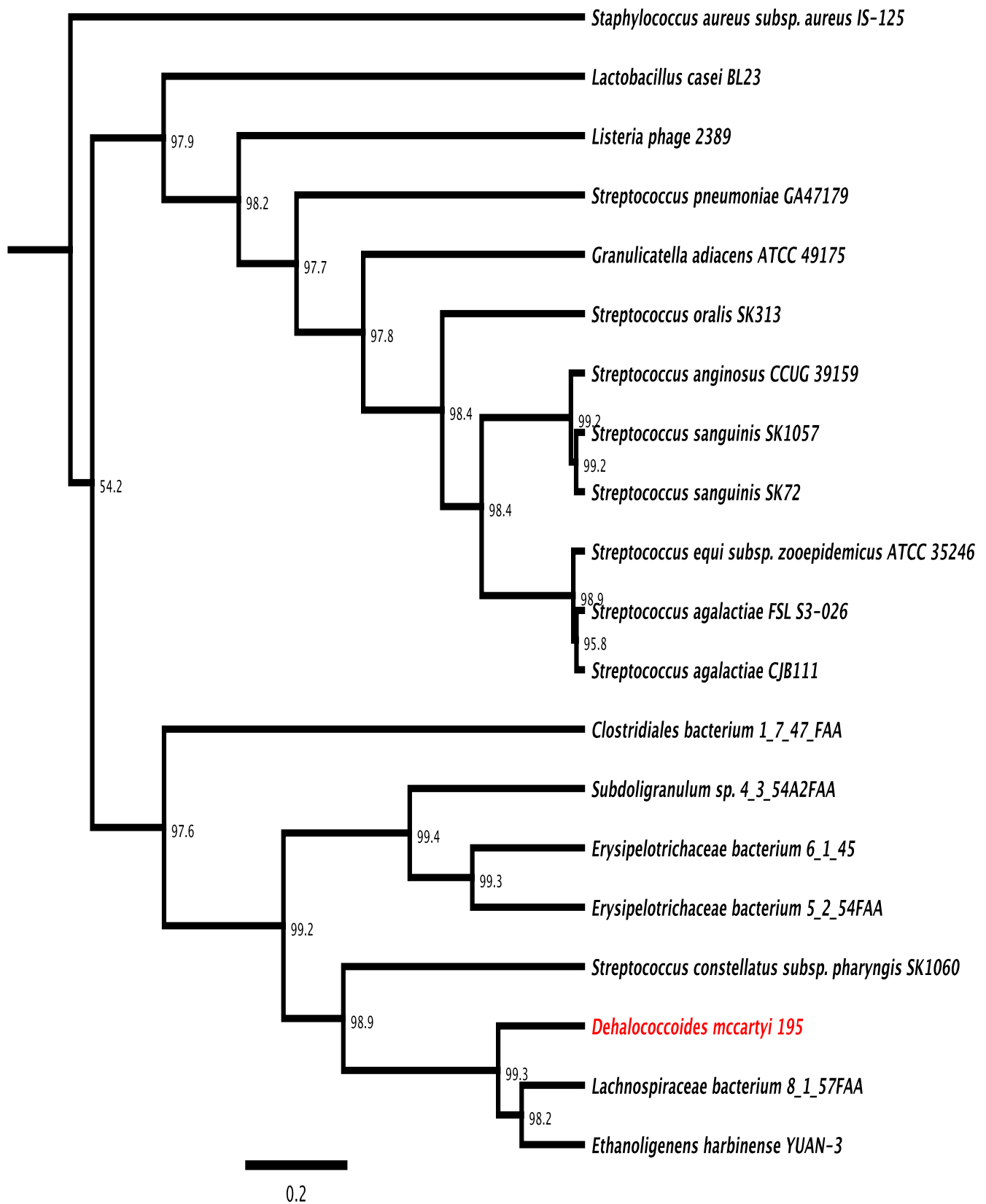


Figure 2.3: Amino acid sequence tree of the phage tail tape measure protein of strain 195 compared to other sequenced prophages, except for the *Listeria* phage 2389.

host chromosome. These genes would be expressed early on in infection (8). Other early genes are the polymerase (DET1100), DNA methylase (DET1092), and ligase (DET1103). These genes are likely to be involved in the remodeling of the phage genome. Late genes are the phage structural genes, and genes involved in packaging the phage genome within the capsid. These late genes are mostly at DET1078-1087, which are annotated as capsid, tail and tail modification genes. As described by Johnson *et. al.* (16), early and late genes show differential transcription rates, which correspond to the metabolic state of the cell (28).

### **Phage Activity**

While sequencing projects have identified many prophages, few have been identified as active phages (6). Visualization of virus-like particles (VLP) by SYBR Green I staining and fluorescence microscopy, allowed for the determination that VLPs were present in the culture of *D. mccartyi* strain 195 (22). This protocol allows for the fast determination of VLP but this enumeration is based on size alone and VLP visualized might not correspond to the actual  $\Phi$ D195 but gene-transfer agents (GTAs) or other cellular debris (7, 29). It is unclear by this protocol alone if  $\Phi$ D195 are the VLPs visualized and not cellular debris; therefore, the prophage was induced using mitomycin C and qPCR analysis was used to track phage gene quantities.

Mitomycin C (MC) induces the SOS response, which is a typical signal for phage induction (2, 7). The qPCR 16S rRNA gene and the phage major tail protein (DET1080) were used as proxies for cell and phage numbers, respectively. Both these genes exist as a single copy in the genome of strain 195, and should be in equal numbers unless there are

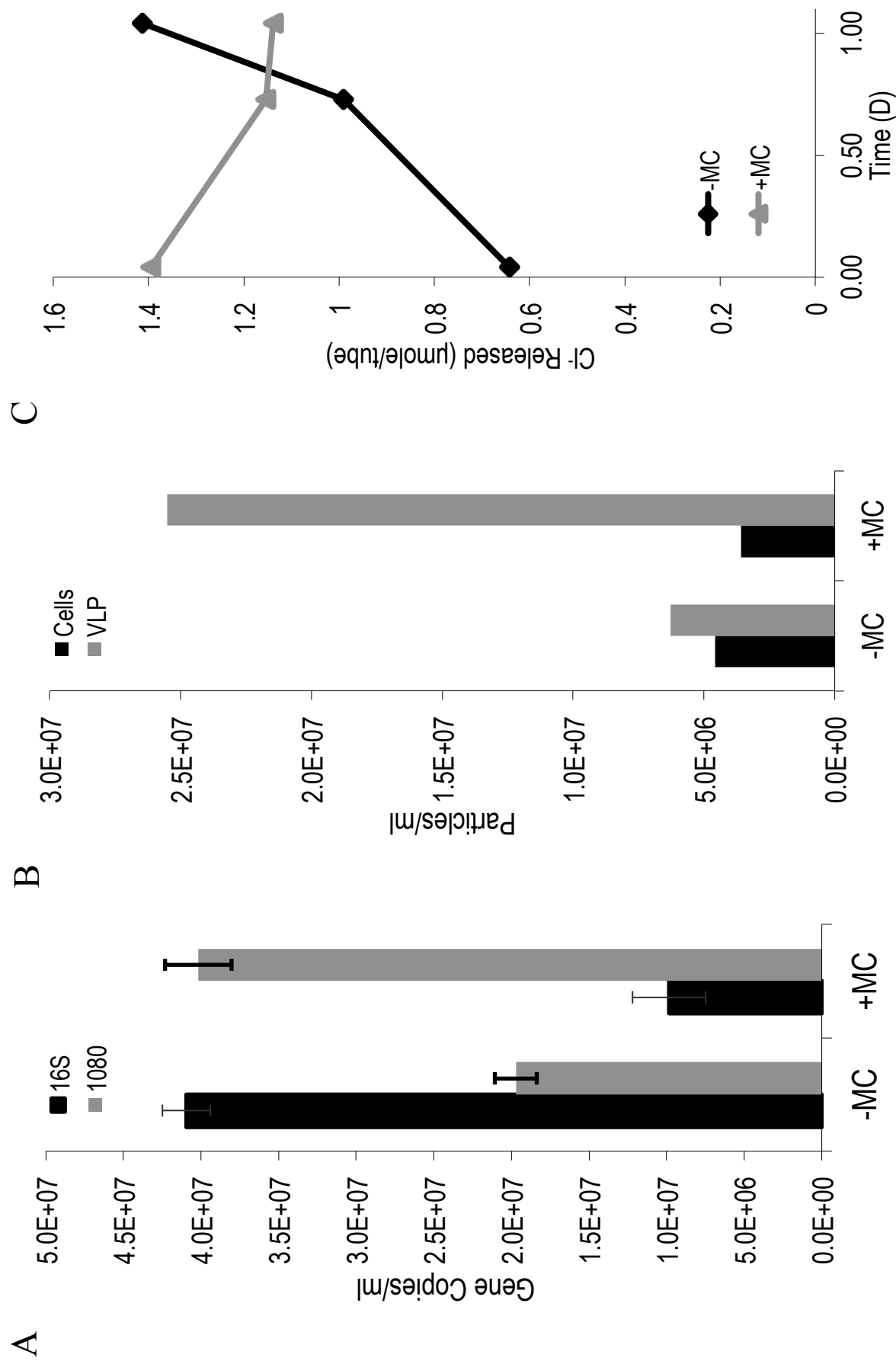


Figure 2.3: 24 hour induction by Mitomycin C in strain 195. (A) Gene counts for 16S and DET1080 (Viral Major Tail Protein), each gene is represented once in the chromosome of strain 195, so that gene counts are equal to cell or viral counts. (B) Microscopy counts by SYBR green staining. (C) Dechlorination by induced and uninduced cultures represented in figures A and B.

additional phage genomes present. DNA was extracted directly from culture medium containing both the phage and bacterial populations at time point to determine their ratio. With the qPCR approach and induction with MC, phage numbers increased from  $1.97 \times 10^7$  to  $4.02 \times 10^7$  gene copies per ml. In the same samples cell number decreased from  $4.09 \times 10^7$  to  $9.8 \times 10^6$  gene copies per ml, resulting in four times as many phage genes then 16S rRNA genes (Figure 2.3). This data suggest that MC induces  $\Phi$ D195 expression and host cells are lysed by viral particles.

These same samples were also stained and enumerated by SYBR Green I staining (22). Although the absolute numbers of cells and VLPs between the qPCR and staining protocols did not agree, the pattern remains the same, an increase in VLP and decrease in cells upon addition of MC, with a ratio of seven VLPs per cell. Taken together, this experiment support the hypothesis that the VLP seen by SYBR Green I staining are indeed  $\Phi$ D195. Upon induction, dechlorination of PCE slowed or stopped (Figure 2.3C), suggesting respiration arrest and cell death. This is further supported by the decrease in cell numbers as determined by microscopy counts and qPCR enumeration.

### **Morphology**

Transmission electron microscopy was used to determine if  $\Phi$ D195 conforms to the typical shape of a *Siphoviridae* with a long non-contractile tail, tail fibers and an icosahedral head (5). Samples were taken from an uninduced culture of strain 195 for TEM analysis. This showed the structure of  $\Phi$ D195 to be very different from that of a typical member of the *Siphoviridae*. The viral capsid appeared to be cigar or spindle shaped and was lacking visible tail fibers. Spindle shaped viruses are members of the *Fuselloviridae* family, which includes characterized phages of *Sulfolobus* hosts (35).



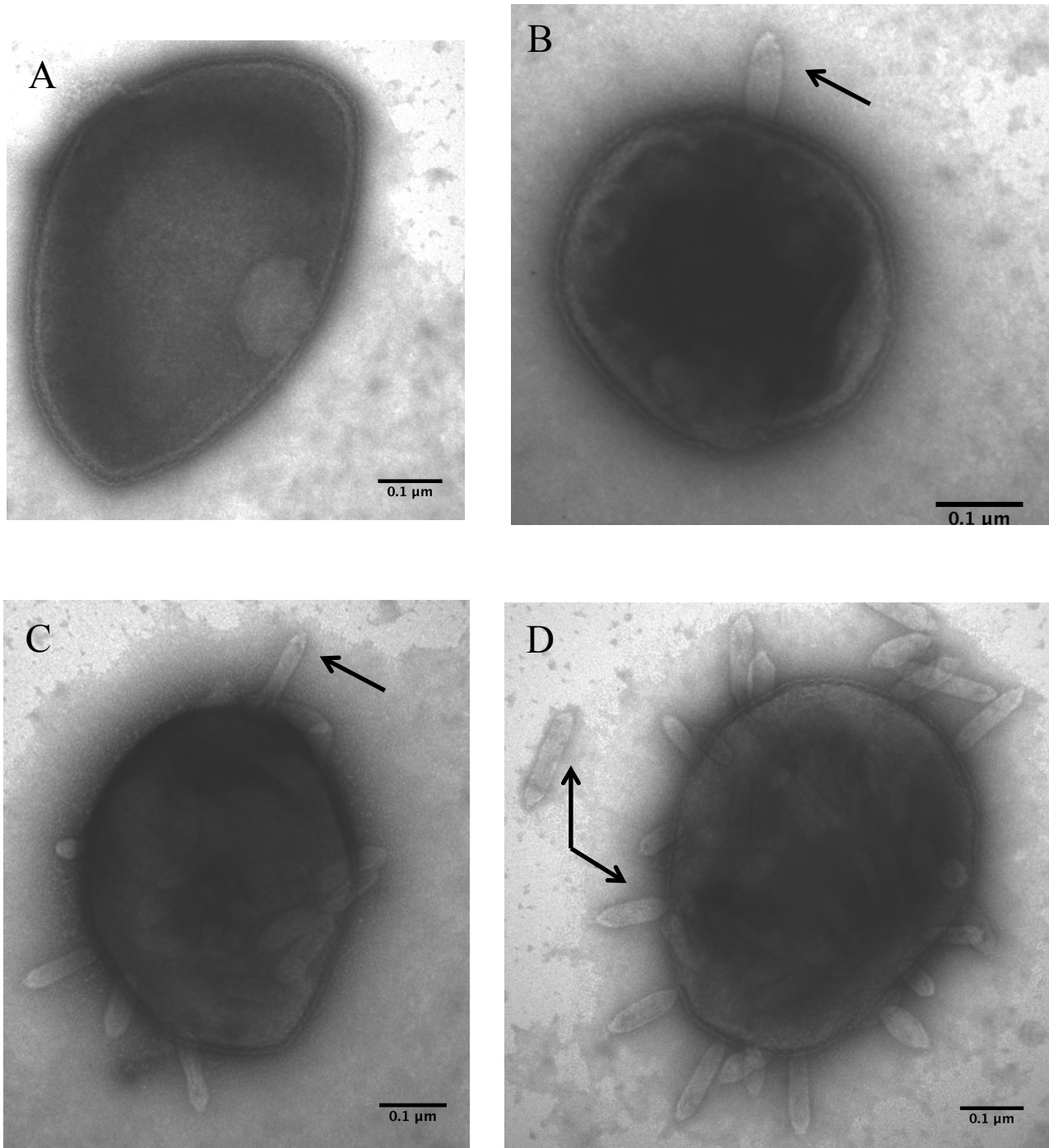


Figure 2.4A-D: Electron micrographs of strain 195 taken showing various stages of infection. EM grids prepared from an uninduced culture and negatively stained with 2% uranyl acetate. A cell showing no signs of infection (A), viral attachment (B) and cell bursting (C-D).

Phages of the *Fuselloviridae* family have a capsid with a typical size of approximately 60nm in diameter with a length of 100nm with a small non-contractile tail at one end of the spindle, has a circular dsDNA chromosome (35, 38). This morphology is similar to that of  $\Phi$ D195, which has a diameter of 20-35nm and length of 80-170nm and does not have a readily noticeable tail. These are also the same size and shape as were noted early on in pure culture of strain 195 (18).

There are two modes for viral particle release from the host cell: bursting and secretion (23). Based on the electron micrographs,  $\Phi$ D195 appears to be secreted. Endolysin, a peptidoglycan degrading enzyme, and holin, a small protein that accumulates within the membrane, are essential proteins for host lysis by bacteriophages (34). While the  $\Phi$ D195 genome encodes for both a holin and an endolysin, the endolysin would be ineffective against the cell wall of strain 195, which does not contain peptidoglycan (27). The apparent secretion of phage particles could be in response to a defective exit stratagem. *Sulfolobus* spp., which also lack peptidoglycan, and *Fuselloviridae* have been described to bud from the host cells rather than cause cell bursting and lysis (3, 26).

## **Conclusion**

Described here is the genomic and activity analysis of  $\Phi$ D195, an active prophage of *D. mccartyi* 195. This genome spans 35.4Kbp and contains 39 ORFs. The majority of these proteins are annotated to be phage related with 13/39 as hypothetical proteins. Further studies would need to be conducted to determine the phage genome physical structure, whether linear or circular.

Experiments presented here show that the prophage is active throughout the life cycle of strain 195 and is inducible under cell stress by mitomycin C. Another

*Siphoviridae*-like sequence has been identified in a *Dehalococcoides* community within close proximity to *tceA*, suggesting that this gene was acquired by a transposable element (33). This is similar to strain 195, which has another insertion element downstream (DET1105-1120) of the prophage. The *Dehalococcoides* prophage present in the KB-1 community spans approximately 30kb, which is smaller than  $\Phi$ D195 by 5.4kb. This phage may represent a mechanism for genetic variation in *Dehalococcoides*, by allowing phage-mediated gene transfer.

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# CHAPTER THREE

## GENOMIC ANALYSIS OF THREE DICHLOROBENZENE DEHALOGENATING *DEHALOBACTER* STRAINS SHOWING SPECIALIZATION FOR GROWTH BY REDUCTIVE DEHALOGENATION

### Introduction

Chlorobenzenes are used in industrial processes such as the manufacture of dyes and pesticides. Dichlorobenzenes (DCB) and monochlorobenzene (MCB) have been released into the environment and are potential human carcinogens. In the environment, these compounds are likely to form dense nonaqueous phase liquids (DNAPLs) and migrate to anaerobic zones in groundwater where aerobic organisms cannot metabolize them to CO<sub>2</sub> (18). Previous work has shown *Dehalobacter* spp. to play a role in the anaerobic dehalogenation of DCB to MCB and benzene (42). Previous studies have shown *Dehalococcoides* to be capable of the dechlorination of higher chlorinated benzenes (1).

*Dehalobacter* spp. (DHB) are obligate anaerobes originally shown to use tetrachloroethene (PCE) as electron acceptors producing to *cis*-dichloroethene (DCE) (21, 56). They have been implicated in the dehalogenation chloroform to dichloromethane, and 1,1,1-trichloroethane to chloroethane (16, 17). Hydrogen and in some cases formate have been shown to serve as electron donors. DHBs are members of the *Firmicutes*, with the *Dehalobacter restrictus* as the type strain (21). DHBs are closely related to *Desulfitobacterium* spp., which are also capable of reductive dechlorination chloroorganic compounds. In contrast to *Dehalobacter*, *Desulfitobacterium* spp. are



capable of using a broad range of electron acceptor including metals and sulfur compounds, and using hydrogen along with organic acids and alcohols as electron donors.

Here we report on the genome of three DHB strains, which are markedly different from their closest known sequenced phylogenetic relative, *Desulfitobacterium hafniense*. These genomes share common genome arrangements and other features, such as genes encoding sporulation, motility, chemotaxis and reductive dehalogenases (rdh). *D. hafniense* strain DCB-2 has five intact rdh genes (28) strain Y51 has two rdh genes (43). The genomes of strains DCB-2 and Y51 are 5.27Mb and 5.72Mb with a GC content of 48% and 47%, respectively. In comparison to the DHBs and *D. hafniense*, the five strains of *Dehalococcoides* have reduced genome size, ranging from 1.34 to 1.47 Mb with a GC content from 47 to 48.9% and 12-36 rdh genes per strain (11). Unlike *D. hafniense*, *Dehalococcoides* can only grow on organochloride compounds and their genomes show metabolic specialization with the presence a large number of rdh genes on a much smaller genome.

A role of DHB for DCB dehalogenation was first demonstrated in microcosms and enrichment cultures (42). Through a dilution to extinction series, strains of DHB, 12DCB1, and 13DCB1, were isolated in pure culture and strain 14DCB1, is the dominant member of a highly enriched culture. Each strain is named for the isomer of DCB it was enriched and then isolated on (41). These three strains have been tested for growth with various substrates, and were only found to use hydrogen or formate as electron donors and chlorinated organic compounds as electron acceptors for growth.

## **Methods**

### **Strain growth & DNA Purification**

All three strains of DHB were grown in 500ml minimal salts medium amended with Ti (III)-NTA and 0.8 mM Na<sub>2</sub>S as reducing agents, a vitamin solution, bicarbonate/CO<sub>2</sub> buffer system, and a single isomer of dichlorobenzene, as previously described (37, 42). The amorphous FeS reductant described previously (37, 42) caused extensive DNA damage during extraction. Dechlorination was used as a proxy for growth and was monitored as a proxy for growth on a Perkin Elmer gas chromatograph (42). DNA was extracted using a phenol/chloroform/CTAB method. TBE/agarose gels were used to check genomic DNA quality. High-quality genomic DNA was then sent to the Cornell Life Sciences Core Laboratory Center for sequencing.

### **Sequencing, Assembly & Annotation**

Genomic DNA was sequenced by the Cornell University Life Sciences Core Laboratory Center with Illumina technology using the paired-end protocol (average fragment length 200-250 nt) with a read length of 2 x 100nt. One lane of Illumina HiSeq was used to sequence tagged DNA from all three strains of DHB. Sequence and quality files were generated by v1.8 of Illumina pipeline software. A total of ca. 110,000,000 reads were generated per strain. Large files were then split into seven smaller files for each paired end. Then data from smaller files were imported to CLC workbench as paired-end reads with distance of pairs determined by mapping raw reads to *Dehalobacter* sp. strain CF in CLC Workbench. The complete genome sequence of *Dehalobacter* sp. strain CF, which reductively dehalogenates chloroform to

dichloromethane, was obtained from S. Tang and E. Edwards and used with permission, as was the sequence from DHB strain DCA, which dehalogenates 1,1-dichloroethane (DCA) to chloroethane. The draft genome of strain CF was assembled using mate-pair pyrosequencing reads and paired-end Illumina sequence data. This allowed for scaffolding and assembly of the contigs (S. Tang and E. Edwards, personal communication).

The Illumina reads were assembled by CLC Workbench using sizing information from mapping to strain CF, using the CLC *de novo* assembler v1.8. Parameters were set to generate the fewest contigs with the largest size. This optimization resulted k-mer length of 22 and in similarity set to 95% over 80% of the read. Assembled contigs of less than 400bp were discarded. *Dehalobacter* sp. strain 14DCB1 was known to contain low levels of a *Desulfovibrio* sp. contaminant. None of the contigs  $\geq 400$  nt appeared to contain sequences from this organism either from BLAST hits in subsequent analyses or from tetranucleotide analysis (Table 3.4).

Contigs from CLC Workbench were then imported to Geneious (v5.6) (9) and further assembled using the Geneious *de novo* assembler. For Geneious assembly, a minimum of 25bp overlap with an 80% identity was required (Table 3.1).

Genome Name	Paired-End Lengths (bp)	Reads	CLC Assembly			Geneious Assembly	
			Longest Contig	Contig #	K-mer	Longest Contig	Contig #
12DCB1	130-350	13,065,516	680,274	78	22	680,510	62
13DCB1	110-275	13,319,332	173,044	121	22	229,964	79
14DCB1	100-330	13,021,098	630,194	70	22	630,448	54

Table 3.1: Assembly statistics from 3 strains of *Dehalobacter* from two different assembly programs, CLC Workbench followed by Geneious.

Once the genomes had been assembled by both CLC Workbench and Geneious, contigs were aligned to strain CF using Mauve (v2.3.1) (46). Contigs were ordered and

oriented to reflect the order when aligned to strain CF. Gaps between some contigs were closed using the Illumina reads (Table 3.1).

After gap closing by using the reads, the resulting sequence was uploaded to the RAST server for annotation (3). Genome maps were drawn with Artemis and DNA Plotter (5, 6). GC skew was drawn with a window size of 10,000 bp and 200 bp step size. GC skew is defined as  $[(G - C)/(G + C)]$  (6). We used the *dnaA* gene to define the origin of replication in all three genomes. Category of orthologous clusters (COGs) (51) were determined by using the Community Cyberinfrastructure for Advanced Microbial Ecology Research and Analysis (CAMERA) (35).

Genes involved in sporulation were identified based on BLAST identity to genes required for sporulation as described previously (39) and RAST annotations.

### **Protein & phylogenetic relationships**

Sequences were aligned first using ClustalW (52). The resulting alignment was then used to create a phylogenetic tree using the MrBayes within Geneious (27).

*Desulfitobacterium hafniense* strain Y51 was used as an outgroup using HKY85 substitution model (23). Chain length set at 1,100,000 with a subsampling frequency of 200. Priors were set with an unconstrained branch length (23). Strain relatedness determination by the 16S rRNA gene identity was calculated as part of phylogenetic tree. Average nucleotide identity and tetranucleotide frequency were calculated as described previously (31, 45).

The proteins annotated by RAST as either a TceA or PceA reductive dehalogenases were used in creating a protein phylogenetic tree based on amino acid sequence. Open reading frames (ORFs) predicted to be less than 220 amino acids in

length were discarded from further analysis. The tree was created using the Geneious tree builder, based on a neighbor-joining algorithm without an outgroup, and bootstrapping at 100. Some *Dehalococcoides* and other known reductive dehalogenases were included for comparisons. Reductive dehalogenase (rdh) amino acid sequences were obtained from the Genbank for non-*Dehalobacter* species.

## Results and Discussion

### Genome assembly, comparisons, and architecture

	Genome		
	12DCB1	13DCB1	14DCB1
Genome Size (bp)	2,885,128	2,994,781	3,062,036
DNA G+C content	44.3%	44.7%	43.9%
Number of Replicons	1	1	1
tRNA genes	54	53	54
rRNA Operons	3	3	3
Coding Sequences	2854	2956	3028

Table 3.2: Genome overview for the three DCB dehalogenating strains of DHB

Assembly of strains 12DCB1, 13DCB1, and 14DCB1 resulted in 62, 79, and 54 contigs, respectively. Based on 16S rRNA gene sequences (Fig. 3.1) DHB strain 13DCB1 was most closely related to the type strain, *D. restrictus* strain PER-K23, and more closely related to strains CF and DCA than were strains 12DCB1 and 14DCB1, which were nearly identical to each other and with strain FTH2, which is implicated in 4,5,6,7-tetrachlorophthalide dechlorination (57). DHB strain CF served as a reference genome for mapping and orientation of contigs after genome assembly of Illumina data (see methods) and it should be emphasized that even though assembled into a single artificial contig, they are still draft sequences in several contigs (Table 3.1). The resulting assembly and annotation resulted in recovery of one 16S rRNA gene copy for each strain, however

previous data showed three copies to be present in each strain (42). The average coverage of coding sequences in our Illumina reads was for 12DCB1, 13DCB1, and 14DCB1 were 450X, 430X, and 412X respectively, and most of the gaps between the DCB utilizing DHB strain genomes mapped to repetitive regions in strain CF.

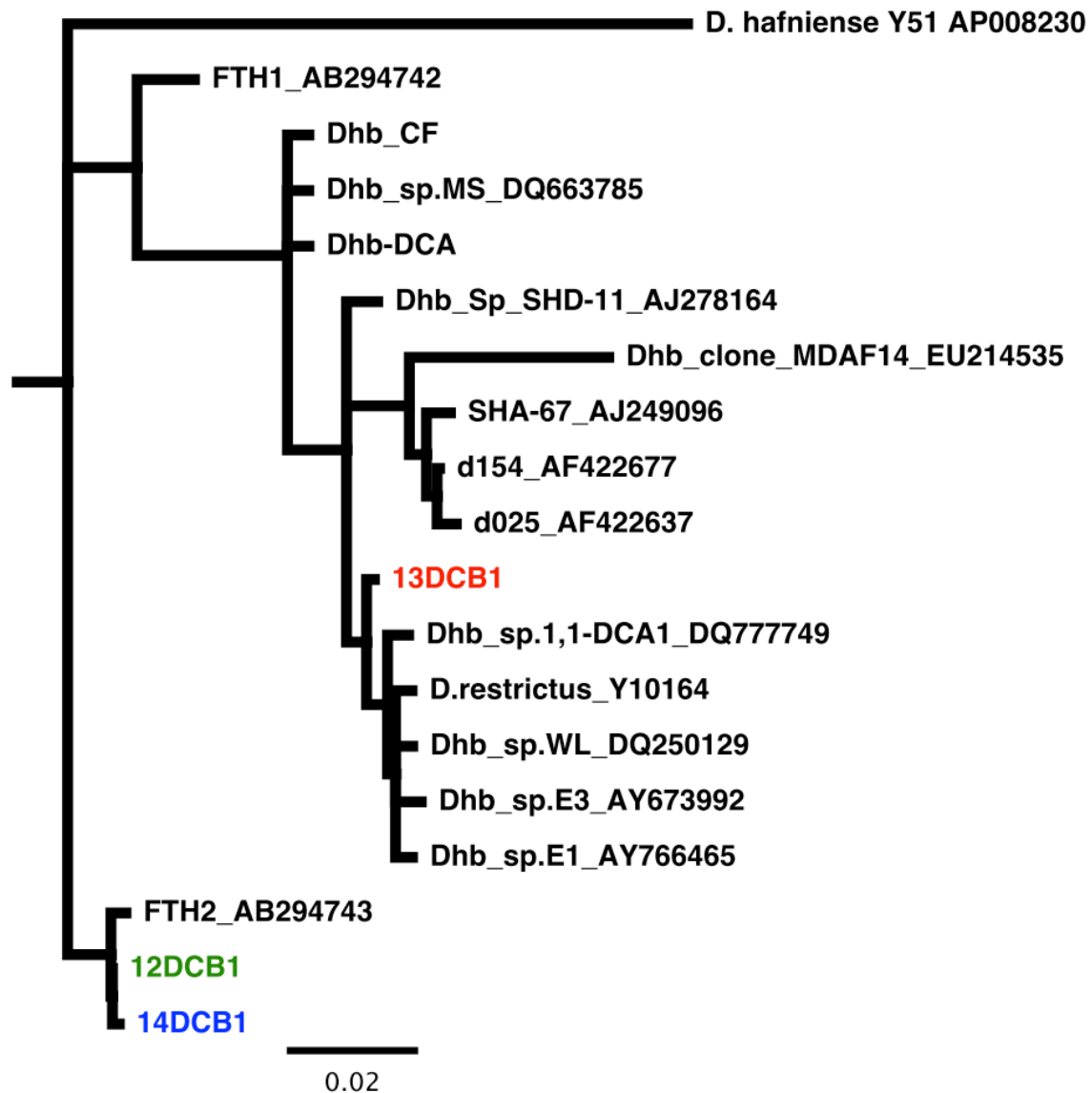


Figure 4.1: 16S rRNA gene tree for DHB strains using *D. hafniense* strain Y51 as an outgroup

Traditionally, a DNA-DNA hybridization (DDH) of >70% between two strains indicated that the two genomes shared a high percentage of DNA similarity and would be

classified as the same species. The subsequent development of 16S rRNA gene sequencing led some to consider a species cutoff of 97% 16S rRNA gene sequence identity (30). There is a 99% identity between strains 12DCB1 and 14DCB1 and a 97% identity between strains 12DCB1, 14DCB1 to 13DCB1 percentage identity as seen in Table 3.3. Strain 13DCB1 has a 99% 16S rRNA gene identity to the reference strain, CF (24).

		16S rRNA Identity				
ANI		DCA	CF	13DCB1	12DCB1	14DCB1
	PER-K23	99.3	99.3	99.8	96.9	96.9
	DCA	-	100	99.4	97.5	97.5
	CF	99.83	-	99.4	97.5	97.5
	13DCB1	96.1	96.27	-	97.0	97.0
	12DCB1	92.49	92.67	92.97	-	99.9
	14DCB1	92.2	92.37	92.82	99.37	-

Table 3.3: 16S rRNA gene and ANI percentages between strains of DHB

Ribosomal RNA genes allow for a quick identification of species or strain relatedness. To address whole genome relatedness, average nucleotide identity (ANI) (29, 31) has been proposed. ANI allows for horizontal gene transfer to play a larger role and it accounts for the whole genome rather than just the slowly evolving 16S rRNA gene. The bacterial species definition by ANI posits that organisms of greater than 94% ANI should be considered the same species, which corresponds to the 70% DDH cutoff. As seen in Table 3.3 the ANI value of strains CF and DCA to strain 13DCB1 is 96.27% and 96.1%, respectively, but strain 13DCB1 was more distant from 12DCB1 and 14DCB1. Strains 12DCB1 and 14DCB1 are nearly identical by ANI, agreeing with their nearly identical 16S rRNA gene sequences. Moreover, many of the predicted proteins in strain 12DCB1 were showed 100% amino acid identity with those from strain 14DCB1 (Figure 3.4), whereas the identity with those from strain 13DCB1 were closer to 95%. A region of

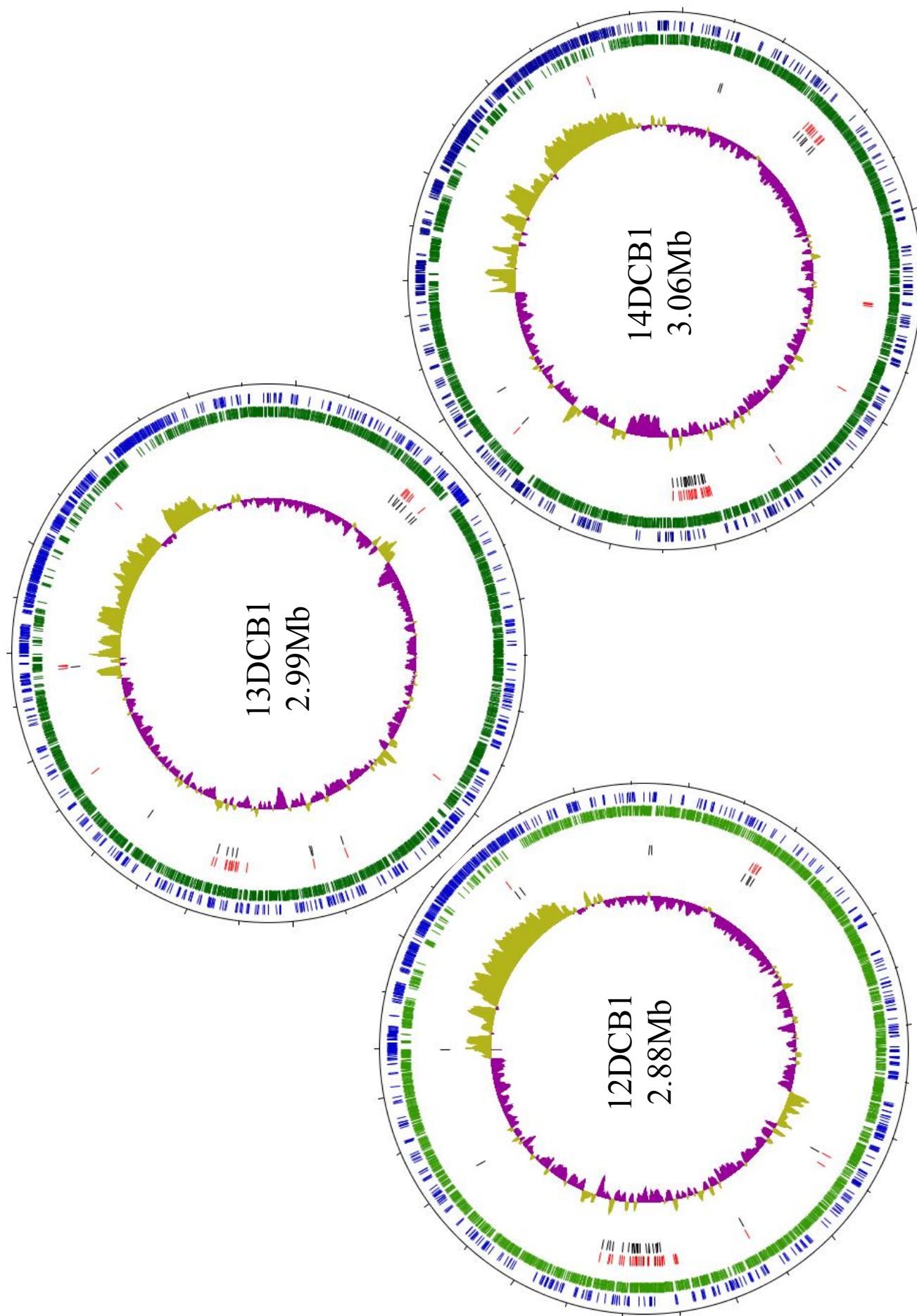


Figure 3.2: Genome Maps for 3 DHB strains. Genes on leading strand are in blue while genes on lagging strand are in green. Putative rdh genes highlighted in red. crp/fnr type regulators highlighted in black. GC skew is in gold and purple. Ticks on outer ring mark every 100,000bp.



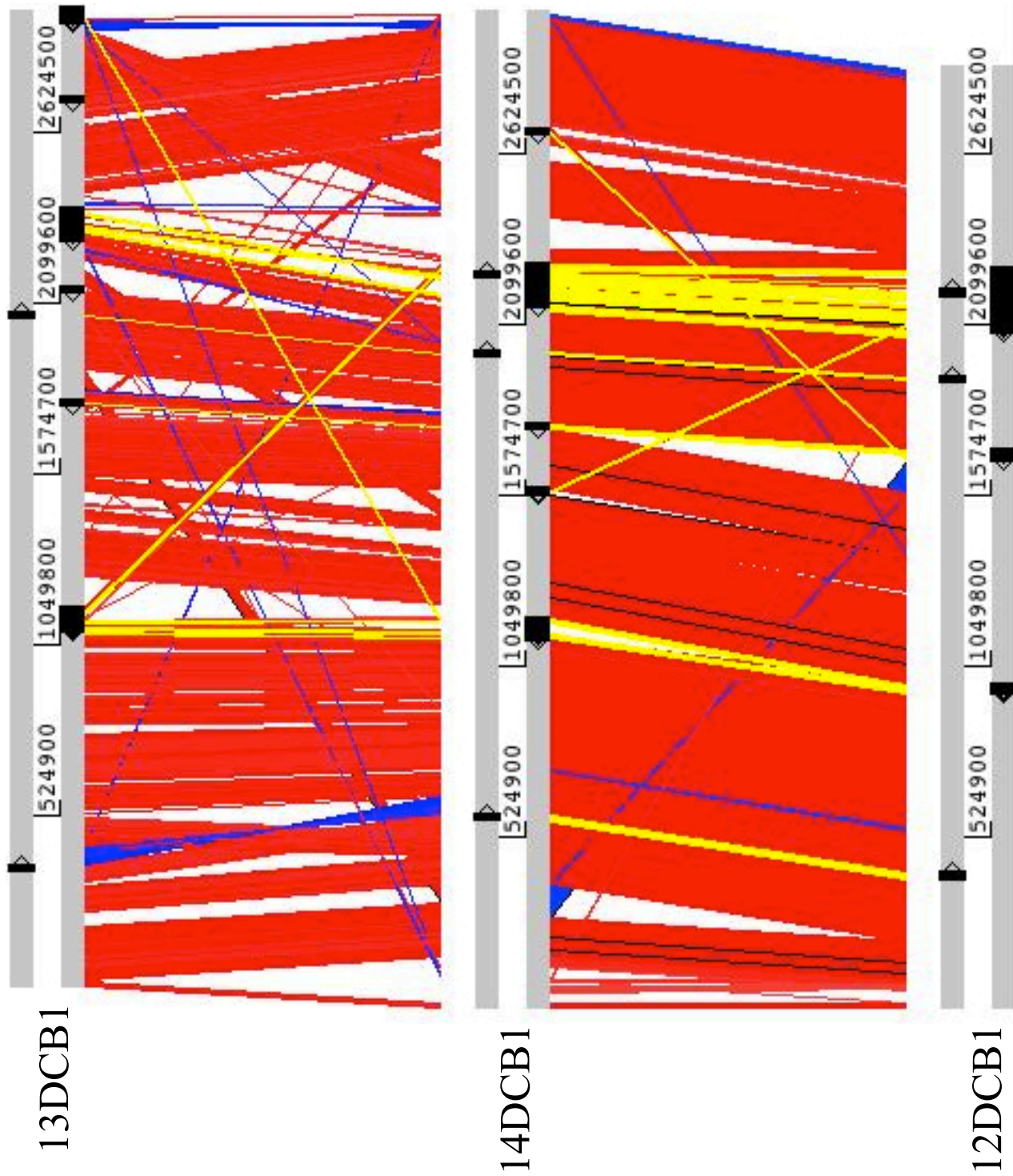


Figure 3.3: ACT comparison between strains 13DCB1, 14DCB1 and 12DCB1 with *rdh* genes highlighted in yellow

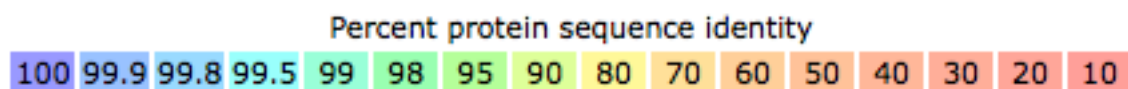
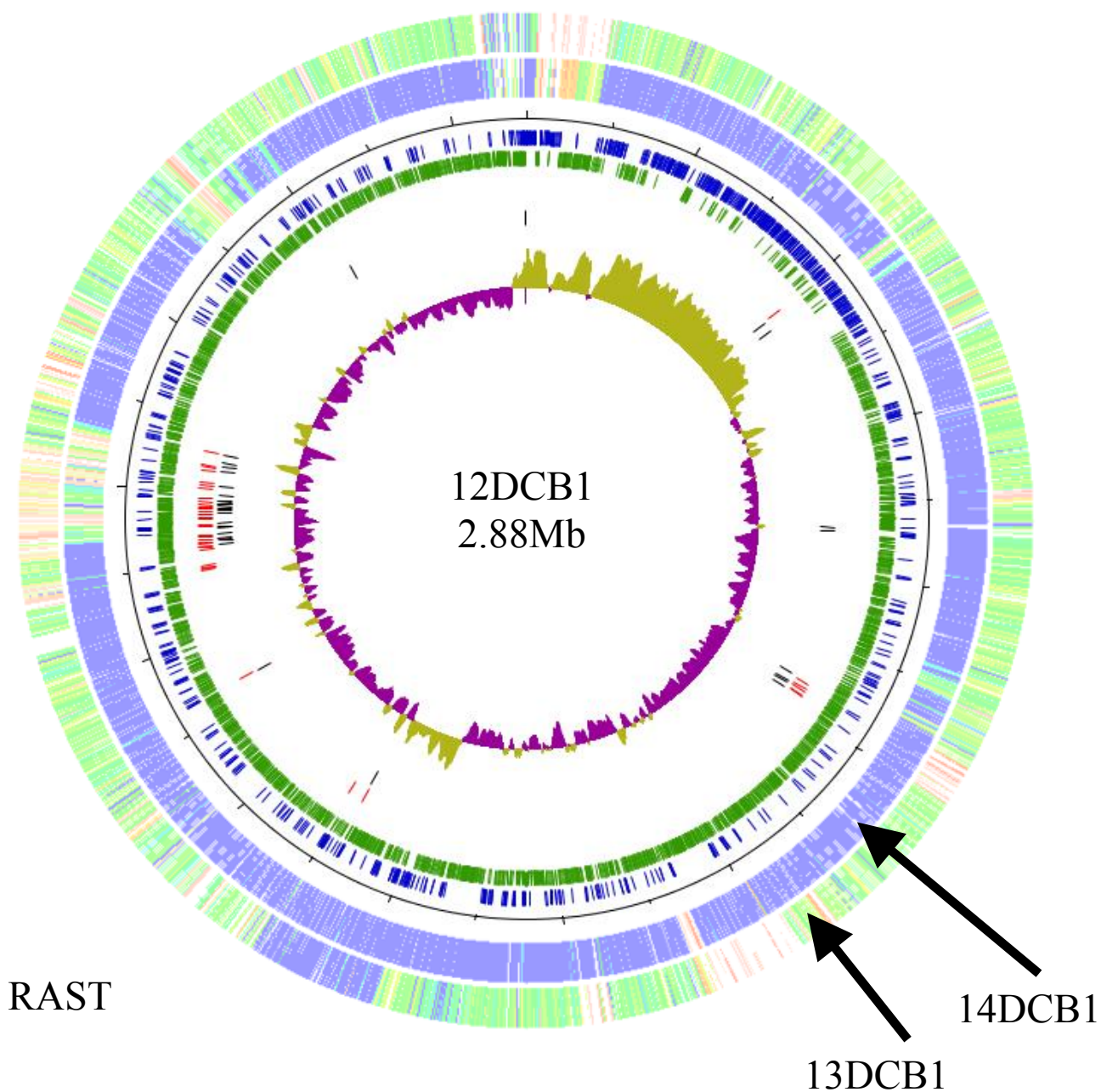


Figure 3.4: Percent protein sequence identity as calculated by RAST of strains 13DCB1 and 14DCB1 compared to 12DCB1. For 12DCB1 genome, genes on leading strand are in blue while genes on lagging strand are in green. Putative *rdh* genes highlighted in red. *crp/fnr* type regulators highlighted in black. GC skew is in gold and purple. Ticks on outer ring mark every 100,000bp.

identity between strains 12DCB1 and 13DCB1 around 200° was populated mainly with unidentified ORFs. Comparisons by tetranucleotide frequency resulted in >0.99 identity among all three strains (Table 3.4).

	13DCB1	12DCB1	14DCB1
13DCB1	---	0.996	0.995
12DCB1	0.996	---	0.998
14DCB1	0.995	0.998	---

Table 3.4: Tetranucleotide identity of the three DCB dechlorinating strains of DHB

Genome mapping resulted in sizes of 2.88Mb for strain 12DCB1, 2.99Mb for 13DCB1 and 3.06Mb for 14DCB1, with GC contents of 44.3%, 44.7% and 43.9% respectively and ca. 3000 CDS per genome (Table 3.2 and Figure 3.2). As predicted by their ANI values, the genomes of 12DCB1 and 14DCB1 have high synteny to each other with 13DCB1 as less syntenous (Figure 3.3). This is recapitulated in the protein percent identity calculated by RAST in Figure 3.4, showing high protein percent identity of 14DCB1 to 12DCB1, and lower identity of 13DCB1 to 12DCB1.

The terminus of replication in *Firmicutes*, specifically *Bacillus subtilis*, has been defined by the *dif* site (48). In *B. subtilis* the *dif* site has been localized to 166° clockwise from the origin of replication and *dif* sites are in the vicinity of 180° from the origin in most *Firmicutes* (20). The bases at *dif* sites are considered conserved throughout the *Firmicutes*. The genomes of the three DHB dechlorinating DCBs were searched and potential *dif* sites were then mapped to the chromosome. Of the potential *dif* sites, the most similar to the *Firmicutes* conical sequence was found at ca. 60° from the origin in each strain, which corresponds to a large switch in GC skew and coding strand preference (Figure 3.2 and Table 3.5) as is typically found in replication termini in *Firmicutes* (15, 53).

Canonical	ACTTCCTATAA TATATA TTATGTAAACT	
Firmicutes Consensus	ACTKYSTAKAA TRTATA TTATGTWAACT	
		Genome Location
Consensus sequence	RCTTWACATAA TATYTA TTATMGGACSC	
D. hafniense Y51	ACTTTACATAA TATCTA TTATAGGACCC	1827925-1827952
D. hafniense DCB-2	GCTTTACATAA TATCTA TTATAGGACCC	2913417-2913444
12DCB1	ACTTAACATAA TATTTA TTATCGGACCC	477224-477251
13DCB1	ACTTTACATAA TATTTA TTATCGGACGC	532411-532438
14DCB1	ACTTAACATAA TATTTA TTATCGGACCC	652205-652232

Table 3.5: Predicted *dif* sequences from DCB dehalogenating DHB strains as compared to *D. hafniense* strains Y51 and DCB-2, and the consensus sequence for *dif* from Firmicutes and the canonical sequence. The consensus sequence for *D. hafniense* and the DHB strains was determined by the identified sequences present in this table.

A similar phenomenon is found in the genome of *D. hafniense* Y51, a phylogenetically close relative of DHB, which has a skew shift and predicted terminus at 80° from the origin. This deviation from a symmetrical chromosome in strain Y51 was attributed to a translocation of 1.22Mb of DNA (28, 43). *D. hafniense* strain DCB-2 has a GC skew shift near 180° which is more typical of *Firmicutes* (28). Based on this gene arrangement in these DHB genomes, this asymmetric GC skew pattern may be a common feature for *Firmicutes*.

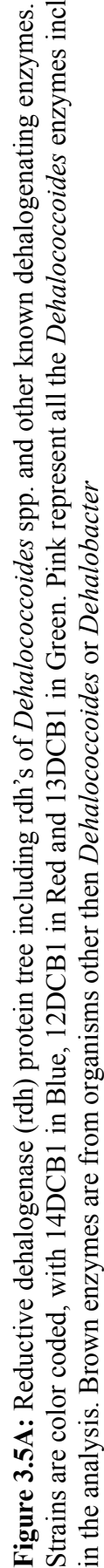
The three DHB strains have similar lifestyles, only known to use H<sub>2</sub> or formate as electron donors and halogenated organic compounds as electron acceptors. The number of proteins per COG category is similar among all three strains (Table 3.6). In comparison to the more physiologically versatile *D. hafniense* Y51, the DHB have fewer coding sequences belonging to metabolic COG categories, specifically lacking in energy production/conversion, carbohydrate transport/metabolism, amino acid transport/metabolism, and secondary metabolite metabolism.

Category	Groups	Functional Classification (NCBI COG)	12DCB1	13DCB1	14DCB1	Y51	D195
Information Storage and Processing	J	Translation, ribosomal structure and	140	139	139	169	127
	K	Transcription	114	127	126	306	49
	L	Replication, recombination and repair	120	149	129	234	85
Cellular Processes	D	Cell cycle control, cell division, chromosome partitioning	34	39	39	48	15
	O	Posttranslational modification, protein turnover, chaperones	80	68	80	105	54
	M	Cell wall/membrane/envelope biogenesis	125	144	127	196	ND
	N	Cell motility	25	28	25	29	ND
	P	Inorganic ion transport and metabolism	80	84	84	224	50
	T	Signal transduction mechanisms	133	107	132	195	43
	U	Intracellular trafficking, secretion, and vesicular transport	39	40	47	36	15
Metabolism	C	Energy production and conversion	219	176	216	479	130
	G	Carbohydrate transport and metabolism	56	56	55	163	34
	E	Amino acid transport and metabolism	141	141	145	258	94
	H	Coenzyme transport and metabolism	100	100	100	162	71
	I	Lipid transport and metabolism	22	32	34	85	23
	Q	Secondary metabolite biosynthesis,	9	10	9	33	4
Poorly Characterized		Multiple function	200	191	203	443	107
	R	General function prediction only	223	232	221	453	119
	S	Function unknown	198	211	205	373	100
		Total	2058	2074	2116	3991	1120

Table 3.6: COG-based Functional categories of *Dehalobacter* strains 12DCB1, 13DCB1, 14DCB1 in comparison to *D. hafniense* strain Y51 and *D. mccartyi* strain 195

The genomes of the obligate organochloride respiring *Dehalococcoides* are severely reduced in size and metabolic capabilities (49), which is analogous but to a lesser extreme in these three strains of DHB. In total, strain 195 has about half as many COGs as the three DCB dechlorinating strains of DHB. When considering the differences in sizes between, the lower numbers of total COGs within the genomes of strain 195 and the genomes of the three DCB dechlorinating strain, the relative membership of each COG is roughly equal. However there are noticeable differences; *D. hafniense* Y51 and DCB-2 and *D. mccartyi* strain 195 contain genes encoding a nitrogenase complex,





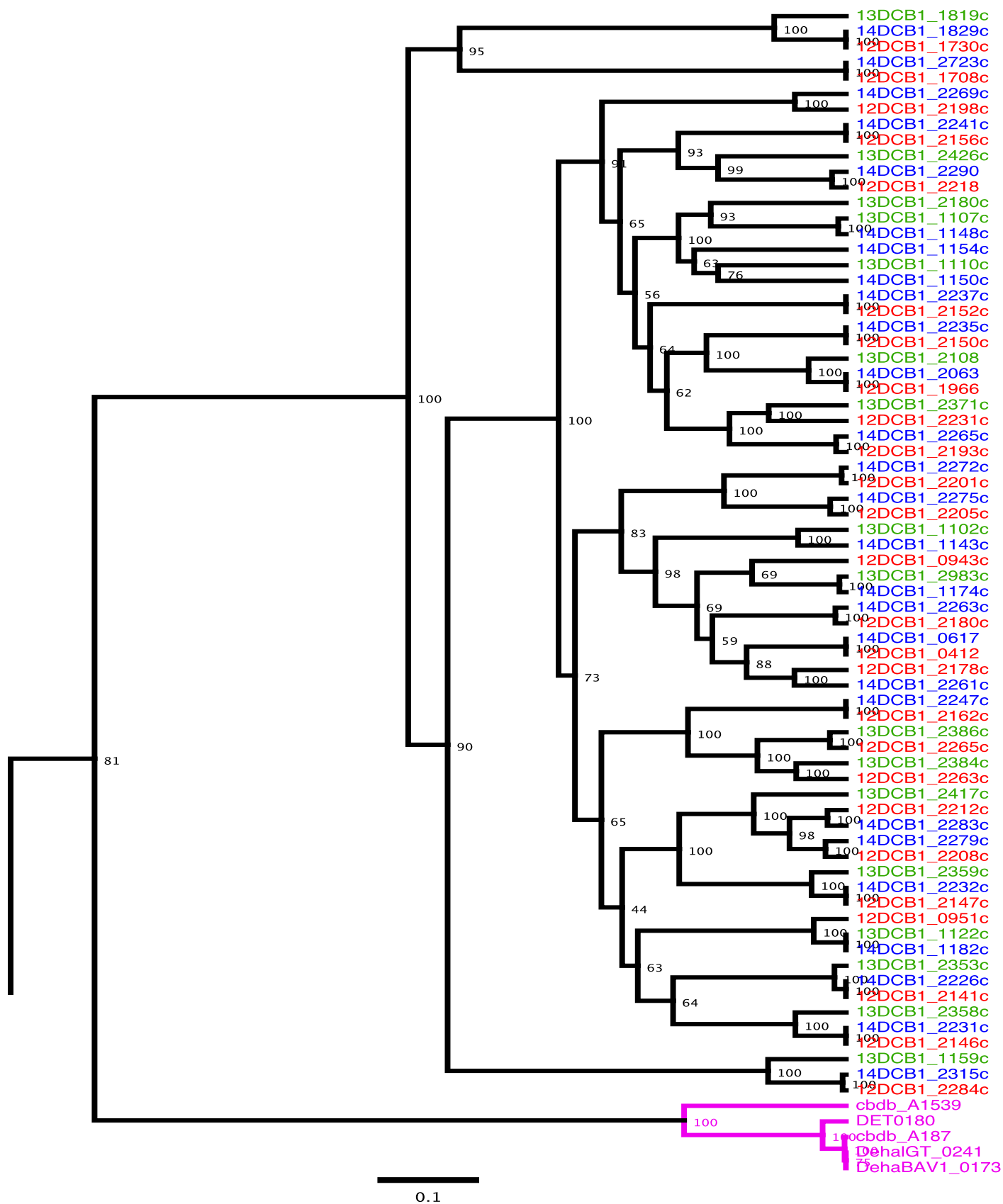


Figure 3.5B: Subset of the rdh tree showing DCB dehalogenating strains rdh genes that are closest to a few *Dehalococcoides* rdh genes

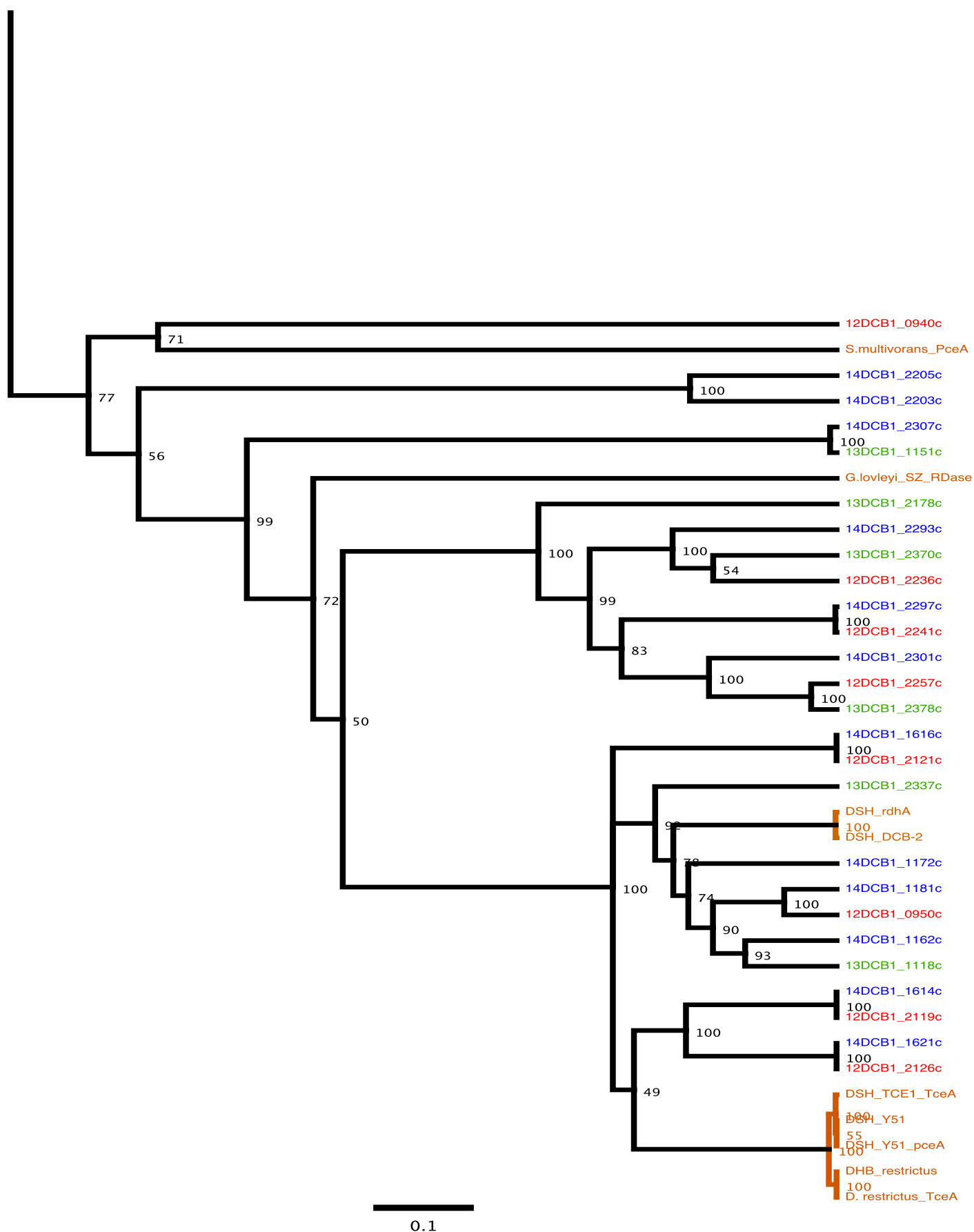


Figure 3.5C: Subset of the *rdh* tree showing DCB dehalogenating strains *rdh* genes that are closest to a few *Dehalobacter restrictus* and *Geobacter* *rdh* genes



whereas DHB lacks these genes, and strain 195 lacks genes annotated for cell wall biogenesis and cell motility.

### **Reductive dehalogenases**

Each strain has multiple genes annotated as *rdhs*, with the typical *rdhAB/BA* structure (50)(Figure 3.5). Strain 14DCB1 has 39 putative *rdh* genes, and is only known to dechlorinate para-substituted chloroaromatics (41). Strain 12DCB can use a variety chloroaromatic compounds with singly flanked chlorines, can reductively dechlorinate PCE to *cis*-DCE and has 39 predicted *rdh* genes. Strain 13DCB1 is the most versatile in known activities, using meta chlorinated aromatics, singly-flanked chlorines, as well as PCE, but has the fewest predicted *rdh* genes with 27. Strains 12DCB1 and 14DCB1 each have one truncated *rdh* whereas strain 13DCB1 appears to have 3 truncated *rdh* genes.

These numbers are considerably greater than those for *D. hafniense* and comparable with those for *Dehalococcoides*. *Dehalococcoides* spp., another genus known to specialize in reductive dehalogenation, have 12-36 potential *rdh* genes with the ranges in extremes in strains BAV1 and VS respectively. A more metabolically diverse organism, *D. hafniense* strain Y51 has two predicted *rdh* genes whereas strain DCB-2 has five. *Geobacter lovleyi* SZ has two predicted *rdh* genes among its diverse respiratory genes. Thus, the two genera most specialized for using chloroorganic compounds have the greatest complement of predicted *rdh* genes, with strains 12DCB1 and 14DCB1 containing even more than *Dehalococcoides*. The function of only a few of these *rdhs* is known in *Dehalococcoides* or DHB (12, 32, 36, 40).

Many of the *rdh* genes in the three DCB utilizing DHB strains are located in two clusters, one at 120-140° and a second larger one at 260-280°, together containing 35/39,

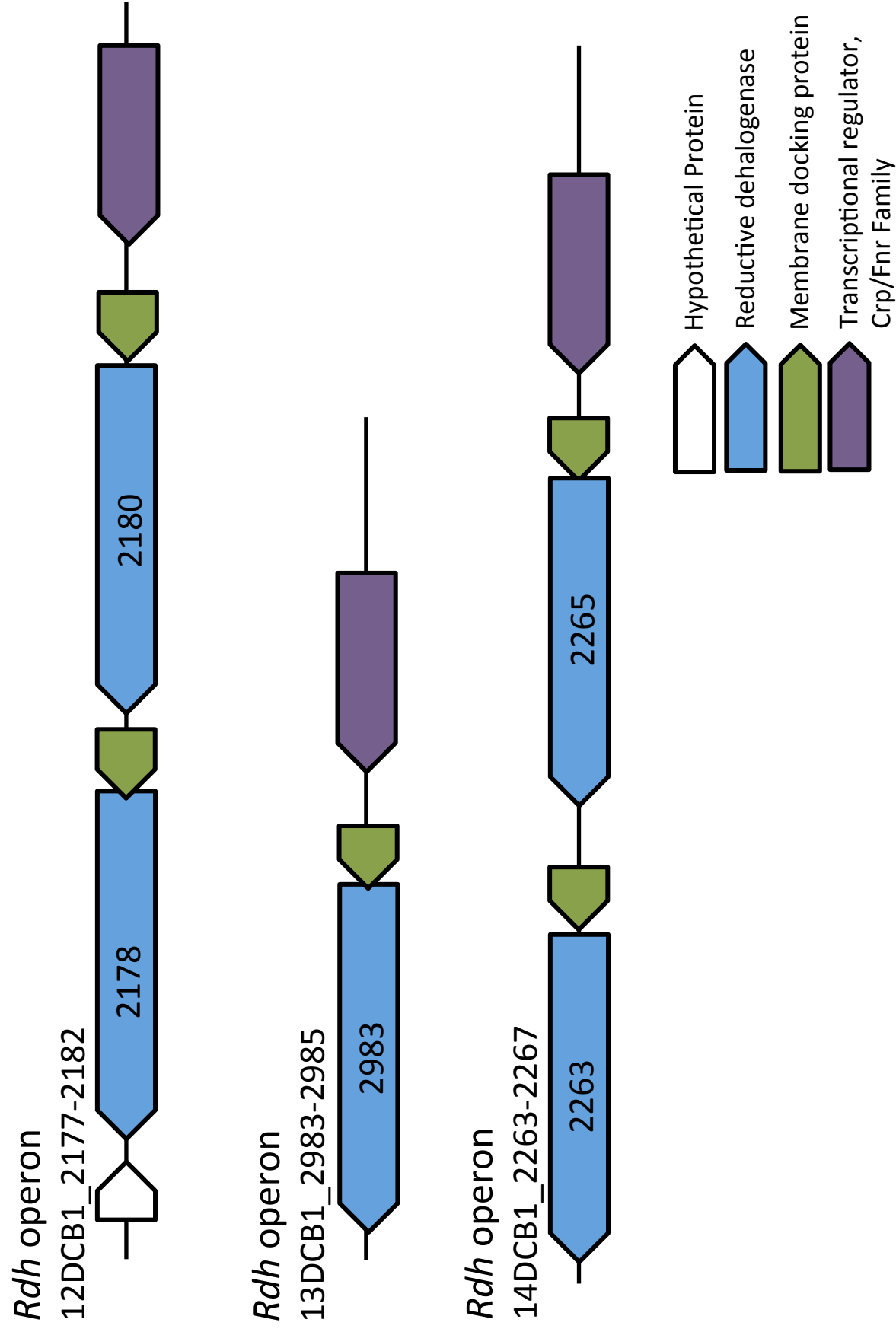


Figure 3.6: Representative operons from each strain containing *Rdh* genes. 12DCB1\_2180 is 77.6% identical by amino acid to 13DCB1\_2983, 80.1% to 12DCB1\_2178, 97.9% to 14DCB1\_2263 and 57.4% to 14DCB1\_2265.

21/27, and 31/40 *rdhA* genes in strains 12DCB1, 13DCB1, and 14DCB1 (Figures 3.2, 3.3). Other smaller clusters of *rdhA* genes are located at other places on the chromosome. Many of the other genes, many predicted to be “housekeeping” genes, in the chromosomes were syntenic (Figure 3.3), particularly between the closely related strains 12DCB1 and 14DCB1. This arrangement is similar to that of *Dehalococcoides* spp. in which there is extensive synteny in the core genome and 91 of 96 *rdhA* genes were located in two so-called high plasticity regions (38), although in Dhc these regions are located on either side of the origin of replication.

The *rdh* genes of the DHB strains fall into distinct clades, (Figure 3.5A-C) with one clade being closely related to known *rdh* of other dehalogenating organisms and another two lineages that are loosely related to *rdh* genes in *Dehalococcoides* and *Geobacter*. The majority of the *rdh* genes of *Dehalococcoides* are contained within their own separate clade. A few of the *rdh* from these DHB strains align closely with *rdhs* from *Firmicutes* known to facilitate the dechlorination of PCE to cDCE (*D. hafniense* Y51 PceA to 12DCB1\_2126, & 14DCB1\_1612 at 67% and 13DCB1\_1118 at 65.4%); which suggests that these proteins are responsible for PCE and TCE dechlorination in DHB, although this will require physiological characterization. Strains 12DCB1 and 14DCB1 share 25 nearly identical (>95% homology) *rdhs*, whereas strains 12DCB1 and 13DCB1 share two and strains 14DCB1 and 13DCB1 share five. One *rdh* is conserved between all three strains, 12DCB1\_2141c, 13DCB1\_2353 and 14DCB1\_2226, which might indicate this is responsible for common dechlorination substrates among these strains (Figure 3.5A-B). Strain 13DCB1 shares only 25% of its *rdh* complement at 95% amino acid

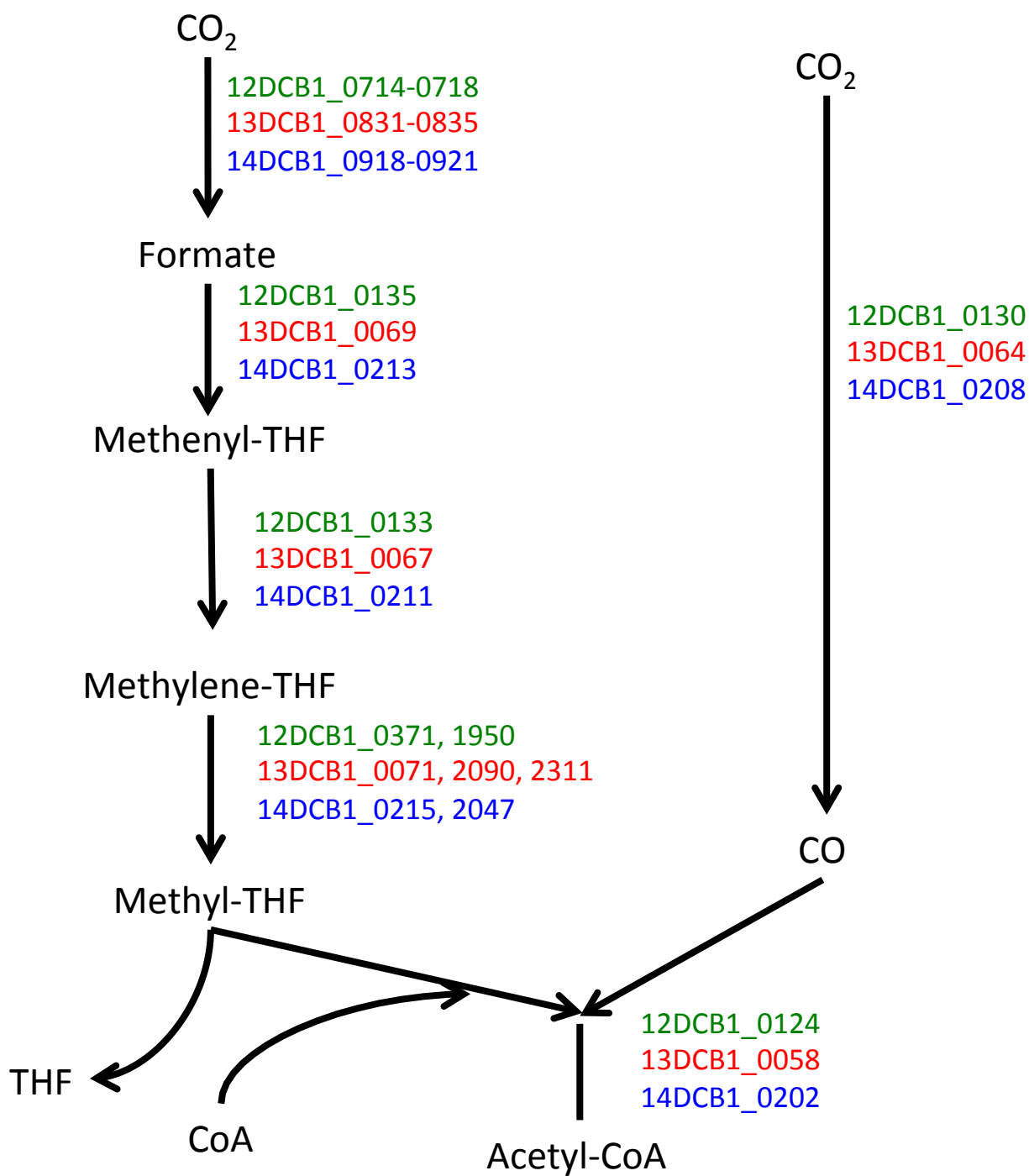


Figure 3.7: Wood-Ljungdahl pathway labeled with locus tags for each strain of DCB dechlorinating DHB

identity with the other two strains of DCB dechlorinating DHBs, yet their genomes are ca. 93% the same.

Reductive dehalogenases of *Dehalococcoides* are located near genes for transcriptional regulation, specifically, two-component signal transduction systems or MarR homologues (49). As shown in Figures 3.2 and 3.6, many of the *rdh* of these DHBs are located next to genes encoding CRP/FNR family regulatory proteins, suggesting that reductive dehalogenation is a highly regulated process in these organisms, but that different regulatory circuits apply. Total numbers of CRP/FNR regulators is less than those of the *rdh* genes, with 34, 18 and 31 in strains 12DCB1, 13DCB1 and 14DCB1, respectively, suggesting that there is overlapping regulation. As seen in figure 3.6, in strains 12DCB1 and 14DCB1, a single CRP/FNR regulator is associated with two different *rdh* genes. This pattern is similar to that found for *rdh* genes of *Desulfitobacterium dehalogenans*. In *D. hafniense* DCB-2 one of the CRP/FNR regulators has been shown to bind DNA and 3-chloro-4-hydroxyphenylacetic acid (13).

### **Electron transport and metabolic capacity**

These three DHB strains use H<sub>2</sub> or formate as electron donors and chlorinated benzenes as electron acceptors. Schumacher and Holliger (47) demonstrated electron transport between hydrogenase and PceA mediated by menaquinone in cells of *D. restrictus*. An examination of the menaquinone biosynthesis pathway in all three strains in this study using the KEGG viewer in RAST indicates that one step is absent. All three strains have an almost complete menaquinone synthesis pathway, but are lacking a gene annotated as 1,4-dihydroxy-2-naphthoyl-CoA hydrolase same as in strain Y51 (Table 3.7). These organisms can grow without the addition of quinones, indicating that the

genes required for quinone or menaquinone biosynthesis are present, assuming these electron transporters are required for growth. All three strains have a number of genes predicted to encode oxidoreductase enzymes, which are annotated as quinone-linked. In *Dehalococcoides* spp. the genes for quinone-ring synthesis are lacking, however, these organisms have been reported to have ubiquinone-8 as their main respiratory quinone with a lower relative amount of menaquinone (49, 55). It has been hypothesized that the respiratory quinones in *Dehalococcoides* could mediate electron transport to the chloroorganic electron acceptor or possibly serve as radical scavengers (55).

Gene Name	Enzyme	Locus tags				
		12DCB1	13DCB1	14DCB1	Y51	D195
MenF	Menaquinone-Specific isochoismate syntase (EC 5.4.4.2)	0884	1014	1083	0517	-
MenD	2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate synthase (EC 2.2.1.9)	0883	1013	1082	0518	-
MenH	2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase (EC 4.2.99.20)	0882	1012	1081	0519	-
MenC	o-succinylbenzoate synthase (EC 4.2.1.-)	0879	1009	1078	0522	-
MenE	o-succinylbenzoyl-coenzyme A synthetase (EC 6.2.1.26)	0880	1010	1079	0521	-
MenB	naphthoate synthase (EC 4.1.3.36)	0881	1011	1080	0520	-
	1,4-dihydroxy-2-naphthoyl-CoA hydrolase (EC 3.1.2.28)	-	-	-	-	-
MenA	1,4-dihydroxy-2-naphthoate polyprenyltransferase (EC 2.5.1.74)	2793	2796	2986	2871	0401
MenG	Demethylmenaquinone methyltransferase (EC 2.1.1.163 )	0241	0290	0443	1907	0398

Table 3.7: Locus tags of genes involved in the biosynthesis of quinones in DCB dehalogenating DHB strains as compared to *D. hafniense* Y51 and *D. mccartyi* 195

One of the predicted oxidoreductases in these three strains is annotated as a selenocysteine-containing formate dehydrogenase (FDH). However, only strain 13DCB1 can use formate effectively while the other two strains' growth on formate was slow and unreliable (41). The predicted FDH large subunit proteins have a UGA stop codon. In the right context this encodes selenocysteine (33). Each strain has a selenocysteine specific tRNA as well as a selenocysteine specific elongation factor (Table 3.8).

Enzyme	Locus Tags		
	12DCB1	13DCB1	14DCB1
Selenocysteine-specific translation elongation factor	2845	2967	3038
Selenophosphate-dependent tRNA 2-selenouridine synthase	0211	0254	0414
Selenide, water dikinase	1096, 1097	1372, 1373	1330, 1331
Formate Dehydrogenase Selenocysteine Containing (EC 1.2.1.2)	0714-0718	0831-0835	0918-0921
Anaerobic DMSO Reductase, Chains A-C (EC 1.8.99.-)	2242-2244	-	2509-2511

Table 3.8: Locus tags for the formate dehydrogenase, selenocysteine related genes, and anaerobic DMSO reductase

These formate dehydrogenases are annotated as NAD-dependent, which is the type associated with CO<sub>2</sub> fixation via the Wood-Ljungdahl acetyl-CoA synthesis pathway. As seen in Figure 3.7, all three DHB genomes are annotated to contain the complete complement of genes predicted to be required for CO<sub>2</sub> fixation via this pathway. This is similar to many other anaerobes including *D. hafniense* DCB-2 and Y51. Autotrophic growth has yet to be observed for the three DCB dehalogenating DHB strains, however growth on CO<sub>2</sub> has been observed by strain *D. hafniense* DCB-2 (28). These three strains of DHB have been maintained on acetate for their carbon source, with CO<sub>2</sub> in their headspaces. Dichloromethane can be fermented by a DHB in enrichment cultures, indicating that DHB might not be limited to growth on chloroorganics (25).

Each strain of the DCB dechlorinating DHBs have the genes annotated as an energy conserving hydrogenase (Ech) complex (Table 3.9). This type of hydrogenase is known as a proton or sodium pump, which can generate low-potential electrons for biosynthetic reactions or reduce the cobalt in the reductive dehalogenases (47, 49).

	Locus Tags		
	12DCB1	13DCB1	14DCB1
Energy Conserving Hydrogenase (Ech), subunits A-F	0625-0630	0717-0722	0827-0832
HypA-HypE	0620-0624	0716-0712	0822-0826
NADH Dehydrogenase (Complex I, NuoABCDHIJKLMN)	2040-2050	2276-2286	2136-2146
NAD-Reducing hydrogenase subunit HoxE (EC 1.12.1.2)	0726	0841	0929
NAD-Reducing hydrogenase subunit HoxF (EC 1.12.1.2)	0725, 1170, 1299	0841, 1462, 1463, 1587	0928, 1403, 1518
Uptake Hydrogenase small and large subunit precursors (EC 1.12.99.6)	1348, 1347, 1489	1694,1695, 2218,2219	1566, 1567, 1715

Table 3.9: Hydrogenases and NADH dehydrogenase of DCB dechlorinating DHB strains

Strain 13DCB1 is unique since it is the only strain that does not contain genes annotated to encode a dimethyl sulfoxide (DMSO) or trimethyl amine oxide (TMAO) reductase complex (Table 3.8). *D. hafniense* DCB-2 and Y51 have been described as having a large number of molybdenum-binding oxidoreductases (28), including DMSO/TMAO reductases. The array of molybdenum-binding oxidoreductases are proposed to give metabolic flexibility to *D. hafniense* DCB-2 and Y51, which is in contrast to the DCB dehalogenating DHB strains. These organisms are more limited and specialized in their options for anaerobic energy metabolism.

### Sporulation and germination

Organisms closely related to DHB, including *D. hafniense* DCB-2, have been observed to sporulate, but sporulation has not been observed in these DHB strains (21). In *Bacillus subtilis* the master regulator Spo0A controls sporulation (8), and controls expression of sigma factors ( $\sigma^E$ ,  $\sigma^F$ ,  $\sigma^G$ ,  $\sigma^K$ ), which in turn mediate sporulation via a signaling cascade. Genes predicted to encode these sigma factors as well as *spo0A* were identified in the genomes of strains 12DCB1, 13DCB1, and 14DCB1. SpoIIIGA is essential in processing  $\sigma^E$ , and was identified in all three strains of DCB dechlorinating



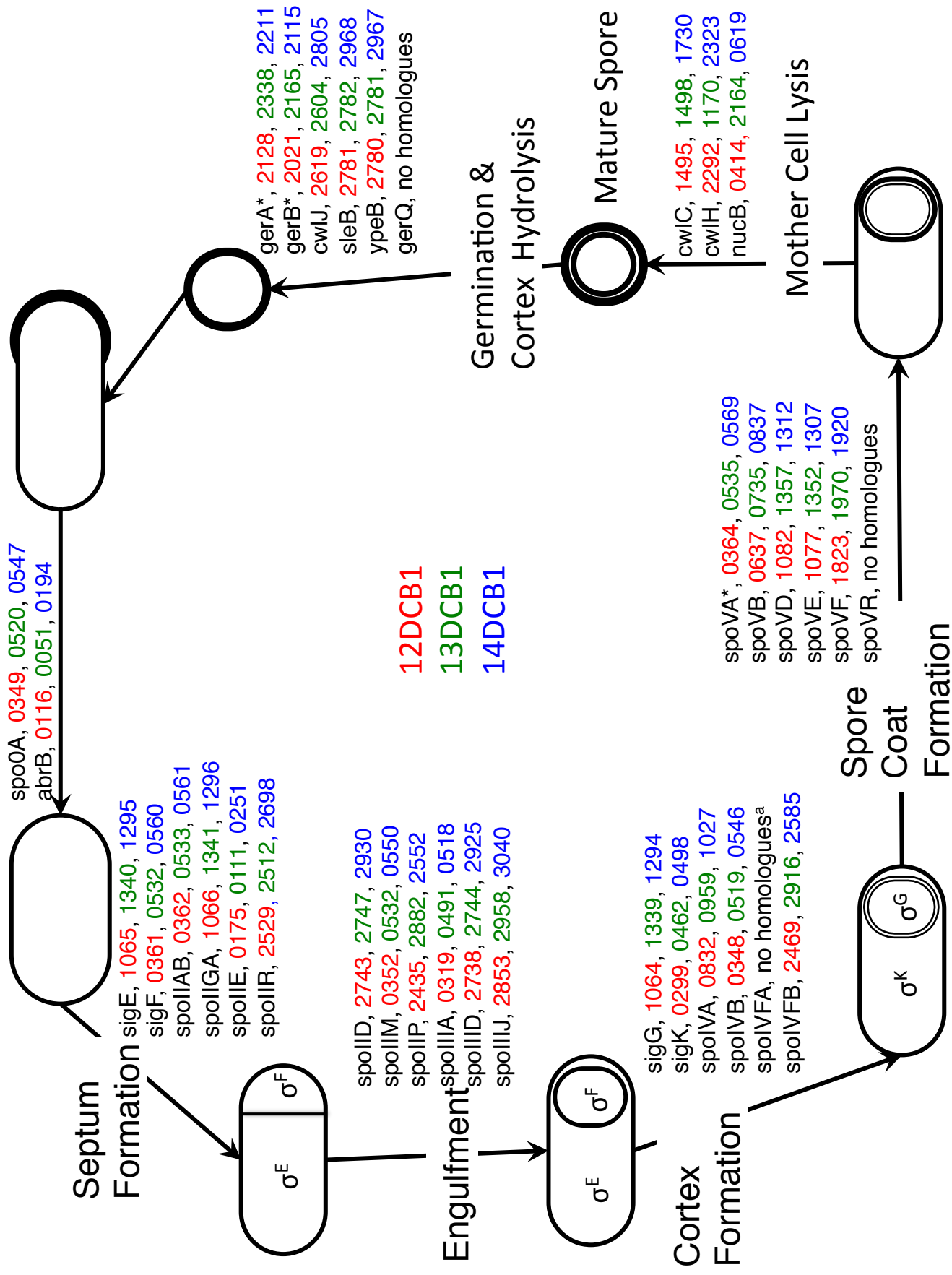


Figure 3.8: Pathway of sporulation and genes involved at each stage

DHBs. *SpoIIR* is present in all three DHB strains, and is required for linking activation of  $\sigma^E$  to the activation of  $\sigma^F$  (26). The mother cell and forespore coordinate activity by using proteins such as *SpoIIR* and *SpoIIGA* (8). These proteins are activated by sigma factors specific to the mother cell ( $\sigma^E$ ) or forespore ( $\sigma^F$ ) (8). These two sigma factors work in concert during septum formation and engulfment of the forespore. *D. hafniense* lacks *spoIIGA* but not *spoIIR*; therefore, the regulatory cascade in early stages of sporulation must be mediated by a different set of proteins in this organism.

After forespore engulfment, the spore cortex is formed and regulated by  $\sigma^G$  and  $\sigma^K$  in the forespore and mother cell, respectively. *SpoIVFB* is a membrane bound protease, which activates  $\sigma^K$ . In *D. hafniense* there are no homologues for *SpoIVFB*, which are present in these three DHB strains (Figure 3.8). *SpoIVFA* is an inhibitor of *SpoIVFB*. The three DHB genomes have ORFs encoding proteins with weak homology to *SpoIVFA* of *B. subtilis*, which are located upstream of the *SpoIVFB*. It is likely that these DHB are using a different signaling pathway then the canonical pathway of *B. subtilis*.

Stage V sporulation genes mediate spore maturation. The *spoVA* genes mediate dipicolinic acid uptake in developing spores (34). The mother cell produces dipicolinic acid, which is essential for resistance to heat, desiccation and UV radiation and spore stability. In *B. subtilis*, these genes are located in a heptacistronic operon *spoVAA-spoVAF* and ending with *lysA* (34). In the DHB genomes, *spoVAA* and *spoVAB* appear to be absent, and the operon starts with *spoVAC* and ends with *spoVAF*. This also holds true for *D. hafniense*. Once the spore coat is formed, the mother cell lyses. This is

mediated by sporulation specific N-acetylmuramoyl-L-alanine amidases, which are activated by  $\sigma^K$  in the mother cell.

Spore germination requires appropriate nutrients and substrates, which are detected by proteins localized to the inner membrane of the spore (22). The levels of the receptor GerA determine the rate at which the spores germinate (14). All three strains of DHB encode for gerA and other conserved germination proteins (Figure 3.8).

It is likely that these three strains of DHB form spores under certain conditions, since they possess genes required for sporulation. Endospores have yet to be observed in cultures of these three strains, even those undergoing long-term starvation. The *abrB* gene is repressed by Spo0A, and is an important protein in the regulation of sporulation and the onset of stationary phase (54). All three DCB dechlorinating strains contain a copy of *abrB*. Sporulation is controlled by interplay of AbrB and Spo0A(4). Spo0A is active when phosphorylated, and environmental signaling of nutrient conditions controls its phosphorylation. KinD is a histidine that has been implicated in environmental sensing in *B. subtilis*. No annotated KinD homologues were present in these three strains of DHB, suggesting a different signal transduction pathway. It is possible that growth in the laboratory does not deplete nutrients enough to trigger sporulation. Most likely some environmental cue is missing in our cultures (similar to experiences with other *Firmicutes* that do not readily sporulate) or the kinases responsible for environmental sensing are absent (10).

## **Conclusions**

At the genome level, there are few differences between each strain, yet they exhibit a range of dehalogenation capacity. This is similar to that seen in *Dehalococcoides*, where phenotypic variation occurs with the spectrum of chloroorganic compounds they are able to dechlorinate (2, 7, 19, 44). These DHBs contain a large number of *rdh* genes, which suggest a larger reductive dechlorination metabolic range than tested. The lack of genes required for nitrogen fixation, fewer total COGs and a reduced genome size in comparison to *D. hafniense* suggests these organisms are streamlining their genomes for growth solely on chloroorganic compounds. Much like *Dehalococcoides*, these DHBs have a reduced genome size in comparison to their closest phylogenetic relatives with sequenced genomes, showing specializing for growth by reductive dehalogenation, and are more likely to be able to cope with environmental stresses (by sporulation) and are able to control location within an environment by motility and chemotaxis. The metabolic specialization for growth on chloroorganics in DHBs is evident from the presence of a large number of *rdh* genes, and growth not observed without hydrogen and a chloroorganic compound. Further experiments are underway to identify the *rdh* responsible for dechlorination of each isomer of DCB within each of these DHB strains.

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## CHAPTER FOUR

### ISOLATION OF AN AEROBIC VINYL CHLORIDE OXIDIZER FROM ANAEROBIC GROUNDWATER

#### **Abstract**

Vinyl chloride (VC) is a known human carcinogen and common groundwater contaminant. VC can be dechlorinated to non-toxic ethene at some contaminated sites. However, VC disappearance without the production of ethene has also been observed. In this study we identify an organism responsible for this observation and conclude that oxygen was present at below detectable limits. This organism, a *Mycobacterium* spp. closely related to known VC oxidizing strains, was present in high numbers in the groundwater sample. Strict anaerobic conditions were maintained throughout the study, which further suggests inadvertent oxygen contamination. This study helps to elucidate observed groundwater contaminate plume dynamics, through the isolation of a strict aerobic organism that may be responsible for disappearance of VC without the concomitant production of ethene.

#### **Introduction**

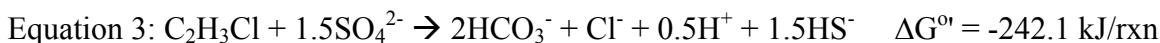
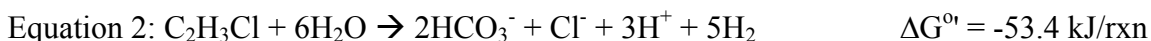
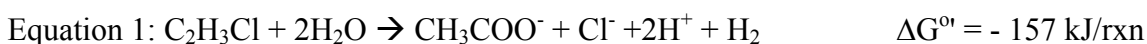
Chlorinated ethenes are a class of toxic organic compounds that are common ground water contaminants. Because of their toxicity the EPA has placed strict limits on the concentrations allowed in drinking water. The least chlorinated chloroethene, vinyl chloride (VC), is considered the most toxic, and, because of this, has the lowest concentration limit of all the chloroethenes in drinking water, at 2 parts per billion (30).

In anaerobic environments, tetrachloroethene (PCE) and trichloroethene (TCE) can be reductively dehalogenated to *cis*-dichloroethene (cDCE), VC, and ethene depending on the organisms present and conditions within the contaminated site (25, 28). *Dehalococcoides* spp. are the only known organisms that can reductively dehalogenate cDCE and VC to ethene, which is non-toxic (22, 24, 32). However, not all *Dehalococcoides* spp. metabolize chloroethenes to the same extent. Strains BAV1 and VS efficiently metabolize VC to ethene, but strains 195 and FL2 only cometabolize VC to ethene and VC accumulates in their cultures (19, 23, 34). When strains like 195 and FL2 are present, incomplete metabolism of higher chloroethenes can occur at contaminated sites, leading to VC accumulation similar to that seen in cultures (25, 30).

At some contaminated sites, VC produced in the methanogenic zone seems to disappear after migrating to anaerobic zones containing other electron acceptors such as sulfate or Fe(III) without the detection of corresponding amounts of ethene; this indicates that VC or ethene is being metabolized by an alternative pathway within the anaerobic zone (7, 25). There are a few theories explaining failure to detect ethene in anaerobic zones, even though VC concentrations are in decline; (1) ethene produced from reductive dechlorination is oxidized to CO<sub>2</sub> as soon as it is produced, (2) VC is directly oxidized to CO<sub>2</sub>, (3) VC is oxidized to acetate which is further metabolized to CO<sub>2</sub> and CH<sub>4</sub>, or (4) oxygen diffuses into the system at below detection limits, and is continually consumed (4, 11, 12, 21). Production of <sup>14</sup>C-acetate, <sup>14</sup>CO<sub>2</sub> and <sup>14</sup>CH<sub>4</sub>, from <sup>14</sup>C-labeled VC has been observed in microcosms (8, 9). However, it remains unknown what microbe might be performing this metabolism (10). Previous studies have reported that VC mineralization could be coupled to Fe(III) reduction and humic substances (5, 6, 13).

Aerobically, VC oxidation has been linked to *Mycobacterium* spp., *Nocardioide*s spp. (16, 40) and *Pseudomonas* spp. (40) among others.

To date, no studies have cultured or identified organisms capable of anaerobic VC oxidation, even though it is thermodynamically feasible with various electron acceptors and despite the evidence from various field and microcosm studies. The conversion of VC to either acetate (Equation 1) or to CO<sub>2</sub> (Equation 2) is energetically favorable under standard conditions with a  $\Delta G^{\circ}$  of -157 kJ/reaction and -53.4 kJ/reaction, respectively. Using a terminal electron acceptor such as sulfate increases the favorability of the reaction to -242.1 kJ/reaction (Equation 3) (38).



To further evaluate the potential for anaerobic VC oxidation and possibly culture a responsible microbe from a contaminated groundwater aquifer, groundwater was collected from a plume with apparent anaerobic VC oxidation activity. This Superfund site in California had overlapping plumes of TCE and dichloromethane. Preliminary studies of groundwater microcosms created from this site indicated anaerobic VC oxidation. We studied these microcosms and cultures further to clarify these results, and concluded that the VC oxidation could be attributed to infiltration of trace amounts of oxygen.

## **Methods**

### **Chemicals**

Most chemicals were purchased from Sigma-Aldrich (St. Louis, MO) at the highest purity available. Gases were purchased from Airgas East (Elmira, NY). Vinyl chloride was purchased from Sigma-Aldrich, 99.5+% purity. Amounts added were calculated from ideal gas law.

### **Microcosms, Enrichment, and Isolation**

Groundwater was collected from a chloroethene-contaminated Superfund site. In the source zone, higher chlorinated chloroethenes were undergoing reductive dechlorination and VC and cDCE accumulated. In anaerobic zones, downgradient from the source, VC disappeared without equalimolar concentrations of ethene being detected, suggesting that VC was being metabolized by anaerobic oxidation (2). Microcosms were prepared by adding 20 g of sediment and 50 ml of groundwater collected from the source zone, to 160 ml serum vials inside an anaerobic chamber. Microcosms were sealed with Teflon-coated red rubber septa. All microcosms received an initial dose of 0.05 ml (ca. 2  $\mu$ moles) VC. These microcosms were incubated inside an anaerobic chamber and were removed for sampling.

Aliquots from one of these sediment microcosms was used to prepare a second-generation of microcosms (hereafter referred to as transfer #1), inside an anaerobic chamber. These second-generation microcosms were inoculated with 0.5 ml of material from the active sediment microcosm plus 99.5 ml of groundwater, in 160 ml serum vials sealed with gray butyl rubber stoppers.

Material from the second-generation microcosms was used as inoculum for a set of enrichment cultures (hereafter referred to as transfer #2). In this case, 0.1 ml was inoculated into 10 ml of mineral salts medium (32) in 27-ml crimp-top culture tubes prepared inside an anaerobic chamber (Coy Industries (Grass Park, MI)). All culture tubes were sealed with Teflon-coated butyl rubber stoppers and removed from the anaerobic chamber. The headspaces of the culture tubes were flushed with 30% CO<sub>2</sub> and 70% N<sub>2</sub> to remove residual H<sub>2</sub> and adjust the pH. The tubes were then amended with 0.1 ml each of sterile anaerobic solutions of 10% (w/v) sodium bicarbonate, 2% yeast extract, and vitamins (33). VC was added, by gas-tight syringe, to the headspaces (0.1ml). Enrichment cultures received 5 mM of anaerobic electron acceptors: sulfate, amorphous Fe(III) oxide, nitrate, or nitrite. Addition of 3 ml of air to the tube headspaces created microaerobic conditions (ca. 3.7% O<sub>2</sub>). The tubes were incubated statically, inverted, and maintained in the dark at room temperature (ca. 20 °C).

Enrichment cultures potentially showing VC utilization were transferred at 1% inoculum to the same mineral salts medium without the addition of yeast extract, for further enrichment and isolation. For isolation of aerobic VC oxidizers under microaerobic conditions, 60 mm plates were prepared with the same medium formulation amended with 1.5% noble agar. Ten ml of the medium plus agar was dispensed aerobically into 27 ml crimp-top tubes, flushed with N<sub>2</sub>, sealed with butyl rubber stoppers, followed by autoclaving. Sterile, anaerobic tubes were then moved into the anaerobic chamber that contained a 50°C heat block to prevent premature solidification. Once tubes reached ca. 50°C, vitamins were added and the molten agar was poured out into the 60 mm plates, which were allowed to solidify inside the anaerobic chamber.

Plates were inoculated, by streaking, from VC oxidizing cultures once they had solidified. The plates were then placed inside a ca. 1 L glass anaerobic canning jar that was then sealed with a lid outfitted with a gas sampling port (3) and were removed from the anaerobic chamber. The headspace was then flushed with a 30% CO<sub>2</sub>/70% N<sub>2</sub> to remove residual H<sub>2</sub>. Following this, 10 ml of VC and 110 ml of air were added through the sampling port to establish microaerobic conditions. Plates were incubated at room temperature in the dark for three weeks. Colonies on these plates were picked, resuspended in 1 ml sterile deionized water, and 0.1 ml was inoculated into 10 ml of the sterile mineral medium described above in 27 ml crimp top tubes, and 0.25 ml VC and 3 ml air were added.

### **Analytical Methods**

For quantitative analysis of VC concentration, 0.1 ml headspace samples were analyzed using a Perkin-Elmer 8500 gas chromatograph with a flame ionization detector (FID) (34). The GC was fitted with a 2m x 3mm stainless steel column packed with 60/80 mesh Carbopack B/SP-1000 (Supelco, Bellefonte, PA) and operated isothermally at 210 °C (35).

Optical densities of cultures were measured at 600 nm on a Spectronic 21 (Bausch & Lomb, Rochester, NY), which was compared to an uninoculated medium blank.

### **DNA Isolation, PCR, and Phylogenetic Analyses**

DNA from groundwater microcosms, and liquid cultures was extracted with a Power Soil DNA extraction kit (MoBio) using the manufacturer's protocol. DNA from isolate was purified using an UltraClean Microbial DNA extraction kit (MoBio) using the manufacturer's protocol. 16S rRNA genes were amplified using universal bacterial



primers 27F and 1492R (1). The PCR reaction contained 1.25 U 5PRIME Taq polymerase, 1X 5PRIME taq buffer, 0.3  $\mu$ M primers, 200  $\mu$ M dNTPs, and 2.5  $\mu$ l of extracted DNA in a total volume of 35  $\mu$ l. The PCR program consisted of 2 min at 95°C, 30 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 1.5 min, then a final elongation step at 72°C for 10 min. PCR products were examined by electrophoresis on 1% agarose gels in TBE buffer.

A clone library of the bacterial 16S rRNA genes in the groundwater microcosms was constructed as previously described (14). PCR products of the 16S rRNA gene from four different VC oxidizing microcosms were pooled for cloning. Cloned DNA was amplified with M13 primers by the same PCR method as above and the PCR products were digested with HaeIII and HhaI (NEB, Ipswich, MA) for restriction fragment length pattern analysis. Digestion mix consisted of 1U of each enzyme, 1X NEBuffer 2 and 10  $\mu$ l of PCR product. Reaction proceeded overnight at 37°C. RFLP patterns were examined on 2% agarose gels in TBE buffer. The samples corresponding to unique patterns by RFLP and VC oxidizing isolate were prepared for sequencing by reaction with ExoSap Enzyme (Affymatrix, Santa Clara, CA) and sequenced at the Cornell University Life Sciences Core Laboratories Center using Sanger sequencing.

Sequences were compared to those in GenBank by BLAST analysis, and related sequences were downloaded for further analysis. The sequences were aligned with ClustalW (39). The phylogenetic tree was created using the Geneious tree builder, based on a neighbor-joining algorithm with JS614 as an outgroup, and bootstrapping at 100, with a 50% support threshold (27).

## **Results and Discussion**

### **Examination of terminal electron acceptors in microcosms and enrichment cultures**

Anaerobic microcosms prepared with site groundwater and sediment, and second generation transfers into groundwater, showed VC disappearance without concomitant formation of ethene, even when not amended with potential terminal electron acceptors (Figure 4.1A); this suggesting that anaerobic VC oxidation was occurring using an endogenous electron acceptor in the groundwater. The site groundwater contained approximately 10.5 mg/L sulfate, <0.1 mg/l nitrate and 2.3 mg/l Fe(II) making sulfate the most likely potential anaerobic electron acceptor. Stalled, unamended groundwater microcosms could be stimulated with the addition of fresh groundwater suggesting that the lacking nutrient or electron acceptor was present within the groundwater (data not presented)(15).

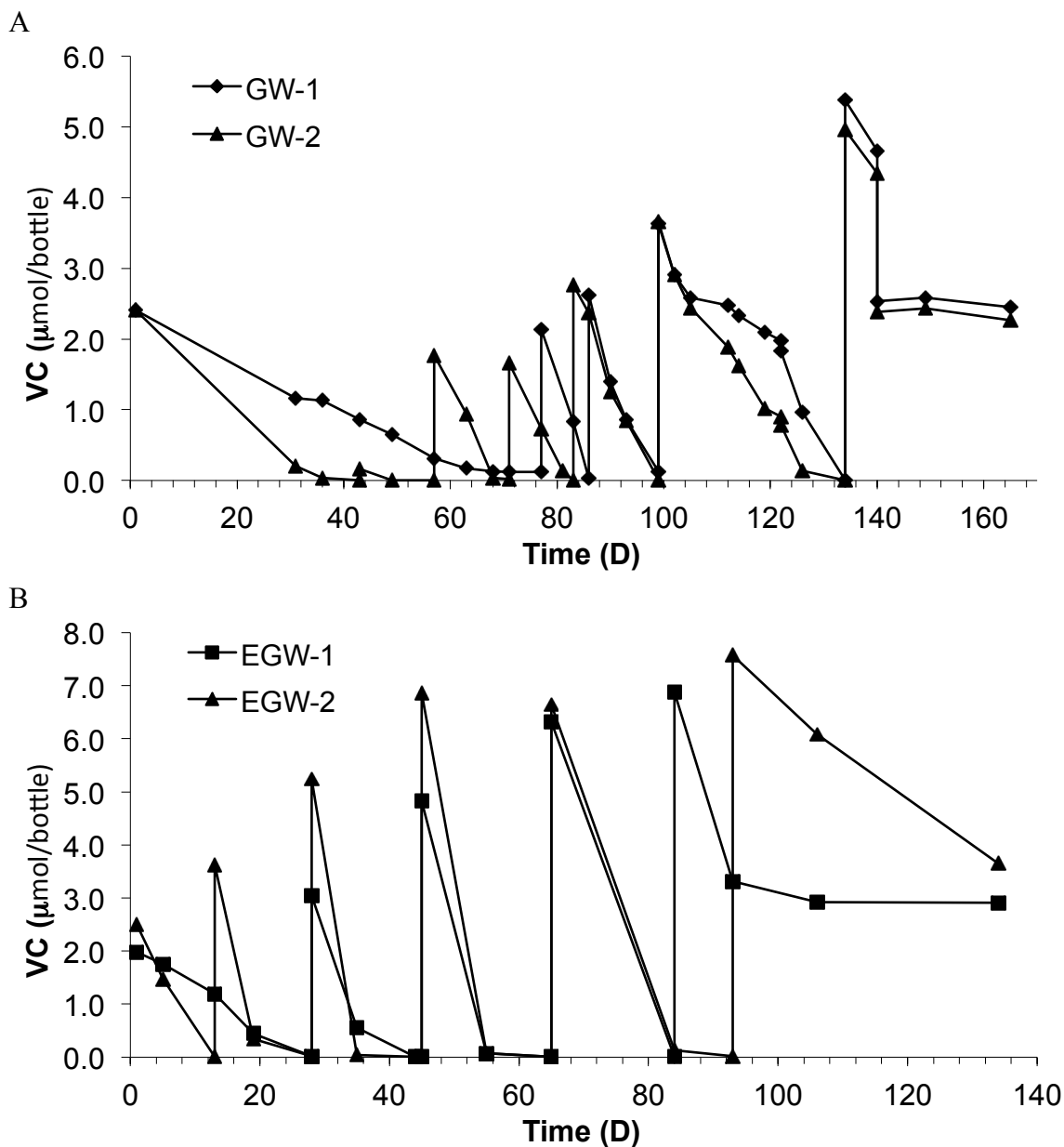


Figure 4.1: VC oxidation in the absence of a terminal electron acceptor in sediment microcosms (GW) (A) and second-generation groundwater microcosms (EGW) (B). Vertical lines indicate additions of VC.

Material from the second-generation groundwater microcosms was used to as inoculum into defined medium for enrichment cultures. These cultures received nitrate (5 mM), nitrite (5 mM), sulfate (5 mM), Fe(III) oxide (5 mM), oxygen (3.7%), or no added

terminal electron acceptor. VC oxidation activity was compared with abiotic controls to account for loss due to sampling and any abiotic reactions.

In the nitrate and sulfate amended and unamended anaerobic enrichment cultures, no oxidation of VC was observed relative to an abiotic control (data not shown). VC loss was only observed in enrichment cultures that received oxygen. Material from the microaerobic enrichment cultures was used to inoculate defined growth medium and these cultures maintained activity through two 1% transfers in liquid medium (Figure 4.2). These cultures also showed VC oxidation at increasing rates, indicative of growth. Using material from these liquid enrichment cultures, semisolid medium plates were inoculated by streaking for isolation and were incubated microaerobically.

After 23 days, flat brownish colonies, ca. 1 mm in diameter, were visible. Microscopic observation showed organisms from the colonies were small Gram-positive rods. Growth in liquid medium inoculated from a colony was slow to begin, taking 86 days to consume the first dose of VC (data not shown). The second dose of VC was consumed within another 12 days. Other organisms capable of growing on VC have been found to show a lag in growth (40). For some isolates, this has been attributed to the absence of vinyl chloride epoxide in cells needed to induce alkene monooxygenase (31).

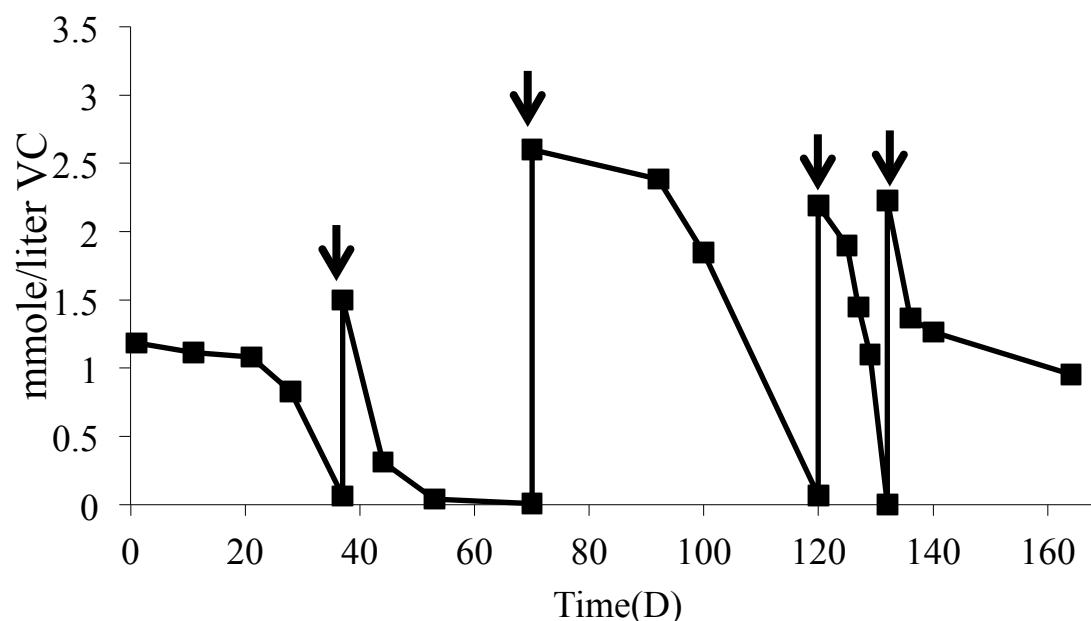


Figure 4.2: Second-generation liquid culture transfer from groundwater microcosms. Disappearance of VC is indicative of oxidation of VC under microaerobic conditions. Arrows indicate addition of VC.

Other VC oxidizing bacteria are also able to oxidize ethene (18). To test if our isolate was able to utilize ethene as well as VC, triplicate tubes were inoculated from a VC grown culture. After 23 days, no VC or ethene remained (Figure 4.3). The consumption of VC and ethene followed an increase in OD concomitant to VC or ethene disappearance. For the second dose of VC or ethene the consumption rate increased along with an increase in optical density. Although the OD is low, cultures unamended with either VC or ethene failed to show an increase in OD. Thus, the increase in OD is growth that is dependent on VC or ethene.

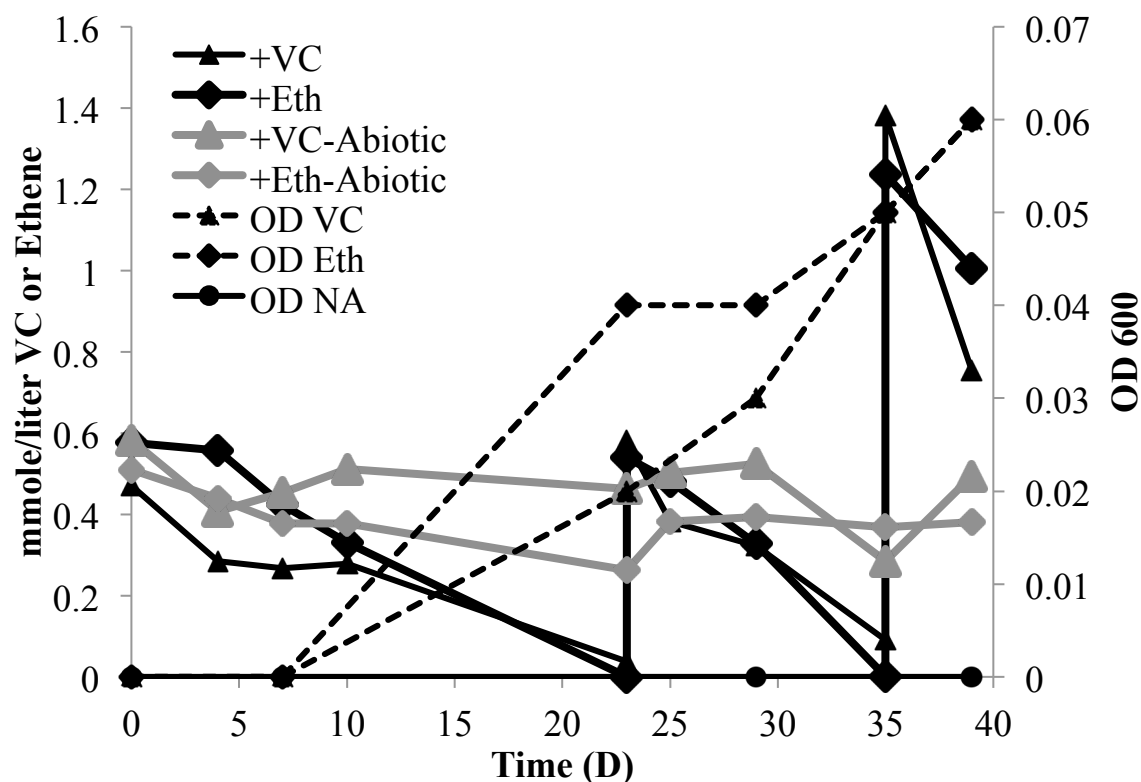


Figure 4.3: Growth of VC oxidizing isolate. Shown are examples of triplicate cultures inoculated from the same source. An increase in OD is concomitant with the oxidation of VC and ethene. Arrows indicated an addition of VC. Grey lines represent abiotic medium controls to account for gas loss during sampling.

### Identification of the VC utilizing organism

An axenic culture from an isolated colony was designated HF26 and its 16S rRNA gene sequence was determined. Comparison to the NCBI database by BLAST revealed that this sequence was closely related to *Mycobacterium moriokanense* and to a known aerobic VC oxidizer, *Mycobacterium* strain JS619 with BLAST identities of ca. 99% over 1400bp and 450bp, respectively (16) (Figure 4.4). *Mycobacterium* spp. are often found in soils and have been shown to degrade a variety of anthropogenic compounds including VC (20, 26, 29, 36).

To determine if the VC oxidation activity observed in the second-generation microcosms was due to organisms similar to this aerobic isolate, a bacterial 16S rRNA gene clone library was constructed with DNA extracted from second-generation microcosms and analyzed for restriction fragment length polymorphisms (RFLPs). One or more representatives of unique RFLP patterns were sequenced. As seen in Table 4.1, a RFLP pattern representing 25 out of the 67 clones had sequences nearly identical with *Mycobacterium* strain JS619 and the VC oxidizer isolated in this study (Figure 4.4).

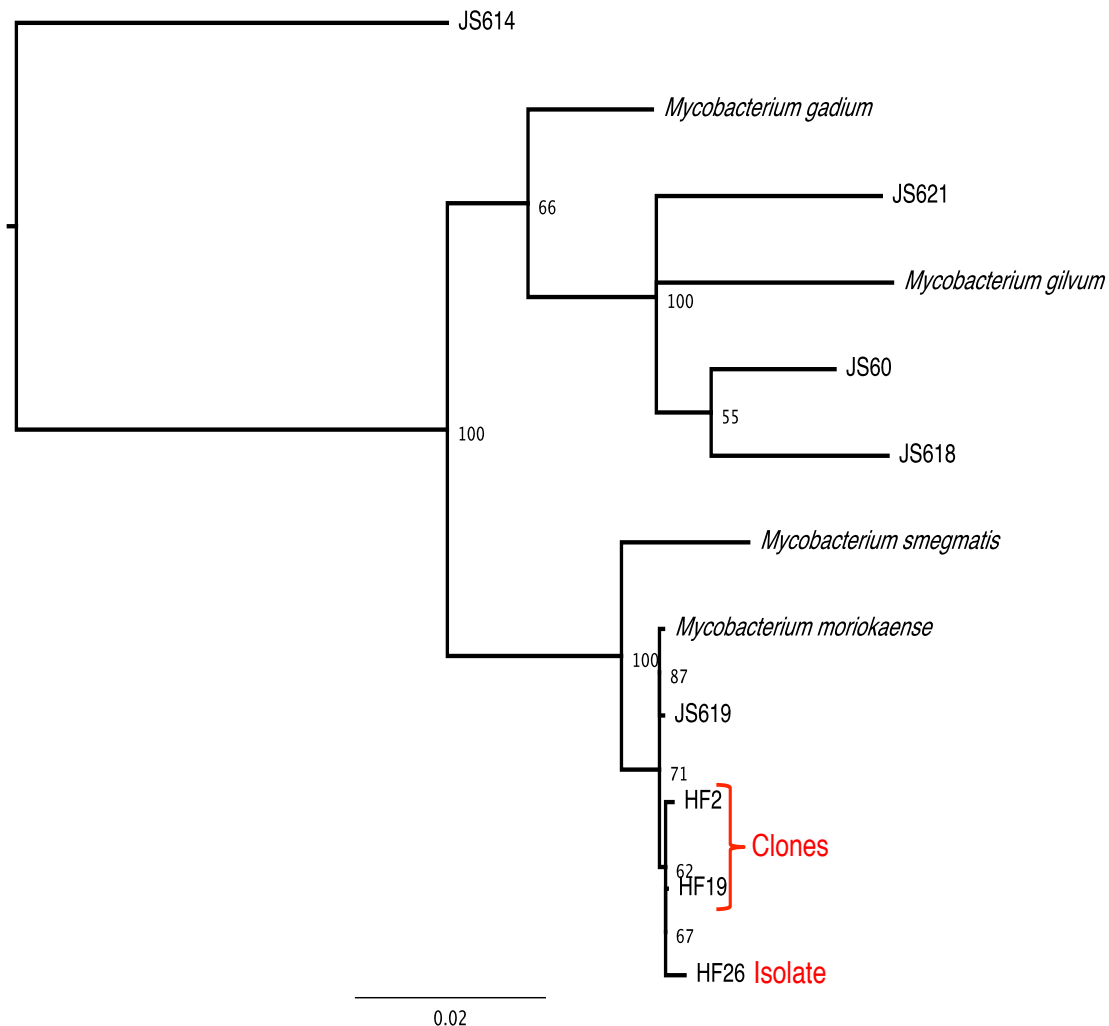


Figure 4.4: 16S rRNA gene tree of aerobic VC oxidizing isolates. Sequence HF26 was obtained from the isolated microaerobic VC oxidizing organism, whereas the other HF strains are sequences from the groundwater microcosm clone library. JS sequences are known VC or ethene oxidizers. Strain JS614, a *Nocardioides* spp., was used as an outgroup in rooting the tree (17).

The 16S rRNA gene analysis indicates that the second-generation microcosms had infiltration of low levels of oxygen, since the dominant member is an obligate aerobic microorganism. The aerobic VC oxidizing *Mycobacterium* spp. most likely scavenged this low level of oxygen, which have been found to scavenge extremely low levels of oxygen (8, 21, 30). Likely sources of this oxygen are repetitive syringe sampling



for GC measurements and/or the passage of bottles with multiple septum punctures through the airlock of the anaerobic chamber when returned from sampling; this could have caused a partial vacuum in the vials, pulling in oxygen still present in early evacuation/flush cycles of the airlock.

The presence of phylotypes related to known anaerobes (Table 4.1) suggests that conditions were anaerobic in the microcosms at least some of the time. In fact, the resazurin added to these microcosms to indicate oxidation/reduction status remained clear throughout the experiments. Moreover, the presence of potentially anaerobic phylotypes prevents us from conclusively ruling out that all the VC oxidation activity was anaerobic, since some of these anaerobes may have participated in VC oxidation.

<b>Best Hit for RFLP type</b>	<b>GenBank Accession Number</b>	<b>Percent Identity</b>	<b>Number of RFLPs</b>
<i>Mycobacterium</i> JS619	AY859686.1	98%	25
UC anaerobic actinobacterium <sup>a</sup>	DQ419604.1	94%	10
UC <i>Desulfobacca</i>	GU472643.1	97%	9
UC <i>Bacteroidetes</i>	JQ580314.1	86%	8
UC <i>Spirochaetales</i> bacterium	EU266876.1	99%	7
UC <i>Chloroflexi</i> (Not Dhc <sup>b</sup> )	HQ183907.1	99%	4
<i>Geobacter pelophilus</i>	U96918.1	98%	4

Table 4.1: RFLP pattern analysis of clones from anaerobic VC oxidizing groundwater microcosms. <sup>a</sup>UC= Uncultured, <sup>b</sup>Dhc = *Dehalococcoides* spp.

## Conclusion

Though initial microcosm studies indicated that VC was oxidized under anaerobic conditions, neither enrichment nor isolation of an anaerobic VC oxidizer was successful. All attempts to transfer material from initial microcosms into defined medium or stimulation of VC oxidation activity in these samples failed. The only activity recovered from the groundwater or original microcosms by culturing was the aerobic oxidization of

VC. An aerobic VC oxidizer was subsequently isolated and identified as being related to a previously described VC oxidizing *Mycobacterium*. This phylotype was also the most numerous in the bacterial 16S rRNA gene clone library from the second-generation microaerobic microcosms representing 38% of the total clones.

Six years after the original microcosms were established, a fresh set of soil samples and groundwater was obtained from the same industrial site and used to prepare nearly 300 new microcosms. During more than three years of monitoring, the only VC biodegradation activity observed in the microcosms was reduction to ethene. This suggests that conditions at the site had become more reducing and favorable to reductive dechlorination over time.

Despite evidence that anaerobic VC oxidation occurs within contaminated sites, the organisms involved have so far evaded enrichment and identification in the laboratory. At these sites, it is possible that oxygen is below the level of detection but high enough to allow for aerobic VC oxidation. Use of Compound-Specific Isotope Analysis can be used to reduce uncertainty about the fate of biotransformed ethenes (37), but cannot fully explain the observed VC loss at contaminated sites. To accurately assess the flux of VC at sites where VC is observed to be decreasing without concomitant ethene formation, detection of known VC-oxidizing phylotypes like *Mycobacterium* spp. within a plume in comparison to non-contaminated groundwater could be indicative of aerobic oxidation of VC at levels of oxygen below detection, as would the ability to enrich aerobic VC oxidizers as we have in this study.

The isolation of this *Mycobacterium* sp. strain highlights the fact that groundwater contamination plumes are dynamic, and our understandings of the biogeochemical cycles

within are not fully understood. Studies which track VC in shallow aquifers show VC mineralization under hypoxic conditions(12), though this is the first study to identify an organism that may be responsible for these *in situ* observations. Further studies must be performed to determine whether these strains of *Mycobacterium* spp. are the organisms responsible for *in situ* VC mineralization.

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## CHAPTER FIVE

### ANAEROBIC OXIDATION OF ETHENE COUPLED TO SULFATE REDUCTION IN ENRICHMENT CULTURES

#### **Abstract**

There have been reports of ethene oxidation to CO<sub>2</sub> or reduction to ethane in anaerobic microcosms, however these reports are very limited and little is known about the environmental fate of ethene under anaerobic conditions or the organisms responsible for such transformation. In this study we report on ethene-oxidizing microcosms under sulfate reducing conditions and development of enrichment cultures. Microcosms were prepared by amending freshwater canal sediments with 0.4 mmol/L ethene and 5 mM SO<sub>4</sub><sup>2-</sup>. Microcosms amended with sulfate depleted the ethene within 74 days and consumed another dose of ethene after 12 days. Production of ethane or methane was not observed. Material from an active microcosm was transferred into mineral salts medium. When inoculated at 1%, ethene disappeared after 63 days with the subsequent ethene dose being consumed after only 18 more days, which is indicative of growth. Further transfers continue to show ethene consumption, consuming multiple doses of ethene along with the concomitant production of sulfide. Quantative PCR showed an increase in total bacterial 16S rRNA gene copies during consumption of ethene. Microscopic observation of these cultures revealed the presence of large irregular cocci, mostly single with some occurring as pairs. Cloning and sequencing from the sixth generation transfer from sediments reveals a dominant phylotype most closely related to *Desulfovibrio adipica* strain TsuA1.



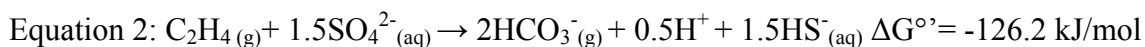
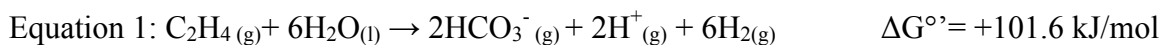
## Introduction

Ethene is a non-toxic, gaseous, organic compound created naturally by plants as a phytohormone (25) and the anaerobic reductive dehalogenation of halogenated ethenes by microbes (26, 28, 40) and is produced in large quantities by the petrochemical industry for use in manufacture of plastics (20). Ethene can stimulate plant responses at very low concentrations; as little as 25 ppb can result in decreased fruit and flower development (34). Microbes present in the soil can degrade ethene under aerobic conditions (11, 13), however anaerobic situations in soils can lead to the accumulation of ethene (18).

Chloroethenes, common groundwater contaminants, can be metabolized to produce ethene (22, 28). Incomplete dechlorination results in production of vinyl chloride (VC) which, can either be dehalogenated to ethene anaerobically (21, 26) or oxidized to CO<sub>2</sub> aerobically (5, 17). Only a few reports describe the reduction of ethene to ethane even though it is thermodynamically favorable with a  $\Delta G^{\circ}$  -98.9 kJ/mole ethene (7, 14, 16). Since reductive dechlorination of PCE and TCE can proceed under anaerobic conditions; ethene will be produced as a by-product if PCE or TCE are fully dechlorinated (15, 16). Because of this, ethene is commonly used as an indicator of bioremediation (30). To achieve mass balance between the disappearance of chlorinated ethenes and the production of ethene, it is assumed that ethene is not degraded anaerobically.

At some contaminated sites, VC seems to disappear without detection of ethene. The failure to detect ethene even though VC concentrations are in decline has been attributed to the direct oxidation of VC under various terminal electron-accepting conditions (6, 8, 9, 39). Alternatively, ethene could be rapidly oxidized as soon as it is produced. The anaerobic oxidation of ethene to CO<sub>2</sub> is thermodynamically favorable only if

coupled to an electron acceptor like sulfate, either directly in a single organism or via syntrophic couplings by interspecies hydrogen transfer. Equations one and two show the thermodynamics of these two types of ethene oxidation.



A previous study showed the conversion of trace amounts of ethene to  $\text{CO}_2$  under sulfate reducing conditions after 160 days (7). In this study we describe the oxidation of ethene coupled to sulfate reduction in a highly enriched microbial culture at much greater concentrations than previously reported and show microbial growth linked to ethene oxidation. As equation 2 shows, the oxidation of ethene is thermodynamically favorable when coupled to sulfate reduction.

Short and long chain alkanes and alkenes are efficiently metabolized aerobically (4, 10, 19). The anaerobic oxidation of ethane, propane and butane as well as long chain alkanes and alkenes has been reported as mediated by sulfate reducing bacteria (19, 24). The known microbially mediated degradation of hydrocarbons has not included the anaerobic degradation of ethene. Investigations into the anaerobic metabolism of ethene will further our understanding of hydrocarbon metabolism and could reduce uncertainty at chloroethene contaminated sites.

## Materials and Methods

### Chemicals

Most chemicals were purchased from Sigma-Aldrich (St. Louis, MO) at the highest purity available. Gases were purchased from Airgas East (Elmira, NY). Ethene was

purchased from MG Industries (Malvern, PA). Amounts added were calculated from ideal gas law.

### **Sediment Samples**

Sediment samples were obtained from Salem canal, New Jersey. Sediment core samples were obtained, and split into one-liter samples, for each 6 inches of the core. These 6 inch samples were then stored in plastic bottles at 4°C in the dark with no precautions to protect them from oxygen. Samples for microcosm studies were taken from below the surface and away from the sides of the plastic bottle. Microcosms were inoculated using the sediment from 8-14" from water sediment interface from a sediment core that was obtained in May 2009.

### **Sediment Microcosms**

Microcosms were prepared inside an anaerobic glove box in 27 ml tubes. To each tube 1 g of sediment (wet wt.) was added to 10 ml of anaerobic deionized water and sealed with Teflon-coated butyl rubber stoppers and aluminum crimps. For inoculated microcosm, 1 ml of slurry from an actively oxidizing microcosm was transferred using a syringe with an 18-gauge needle into microcosm containing 1 g fresh sediment into 9 ml of deionized anaerobic water. The headspaces of all microcosms were flushed using 70% N<sub>2</sub> / 30%CO<sub>2</sub> (Airgas, Certified Standard purity) to remove H<sub>2</sub> from the headspace atmosphere, and ethene was added by gas tight syringe. Unless stated otherwise, all solutions were prepared anaerobically by flushing with high-purity N<sub>2</sub> gas. Microcosms were amended with 1.0g/L sodium bicarbonate as a buffer and vitamin solution (28). Electron acceptors were tested by amending sediment microcosms with 5 mM sodium sulfate, 5 mM sodium nitrate or 5 mM

amorphous iron oxide. Microcosms were incubated inverted and statically at 30°C in the dark.

### **Enrichment Cultures**

A mineral salts medium was dispensed into 27 ml tubes or 120ml vials, 10ml and 50ml, respectively inside an anaerobic glovebox (Coy Laboratory Products (Grass Park, MI))(3). Tubes and vials were sealed with Teflon-coated butyl rubber stoppers and aluminum crimps and removed from the glovebox and autoclaved. All headspaces were flushed with N<sub>2</sub>/CO<sub>2</sub> (70%/30%) to remove H<sub>2</sub> present in the glovebox atmosphere. All tubes and vials were amended with sterile, anaerobic 1.0 g/L sodium bicarbonate, 5 mM sodium sulfate and the addition of a vitamin solution (29). Based on previous enrichments (33), 1 mM amorphous FeS was used as the reducing agent. No other source of carbon was added to the enrichment cultures.

### **Microscopic analysis**

From the sixth generation enrichment culture, 0.3ml was removed and mixed with 10 µl of acridine orange (0.05mg/ml) and incubated at room temp for 2 minutes. The mixture was then centrifuged at 13,000rpm for 2 minutes to pellet the cells, which was resuspended in 50ul of 1X PBS then applied to a 1% agar-coated slide.

### **Analytical Methods**

Sulfide production was monitored using a colorimetric assay in which sulfide is reacted with N,N dimethylphenylenediamine which is then further oxidized by Fe (III). Absorbance was measured at 670 nm on a DU730 LifeScience UV/Vis Spectrophotometer (Beckman Coulter, Indianapolis, IN), which was calibrated to an uninoculated medium

blank. Both headspace and liquid samples were taken from the enrichment cultures to determine sulfide concentration, and compared to standards of a known concentration.

Ethene was detected by headspace analysis (0.1 mL samples taken with a 0.25 mL Precision Sampling Pressure Lok syringe) using a Perkin-Elmer 8500 gas chromatograph with flame ionization detector fitted with a 2m x 3mm stainless steel column packed with 60/80 mesh Carbopack B/SP-1000 (Supleco, Bellefonte, PA) with helium as a carrier gas (30mL/min) (27). Both detector and injector temperatures were held isothermally at 215°C. Compounds were identified using retention times of chemical standards and peak areas were calculated using Peak Simple software. Calibrations based on aqueous standards with the same liquid and headspace volumes as the samples and over the concentration ranges were measured (mmol/L of liquid volume), not by aqueous concentration.

#### **DNA Extraction, 16S rRNA Gene Clone Libraries and Phylogenetic Analyses**

DNA extractions were from 0.5 mL of enrichment culture. Samples were centrifuged at 13,000rpm for 2 minutes and supernant was removed. From this, cell pellets were resuspended and DNA was extracted using the UltraClean Microbial DNA Isolation Kit (MoBio, Carlsbad, CA) following manufacturer's protocol.

A 16S rRNA gene clone library was constructed with amplifications from DNA extracted from the sixth generation ethene enrichment cultures using the universal primers 27F and 1392R (1). The PCR reaction mixture contained 1.25U 5PRIME Taq polymerase, 1X 5PRIME Taq buffer, 0.3μM, 200μM dNTPs, and 2.5μl of extracted DNA in a total volume of 35μl. All PCR reactions were initiated a two minute 95 °C step followed by 30 cycles of 95 °C for 1 min, 50 °C for 1.5 min, and 72°C for 1 min followed by a 10 min hold at 72 °C and a hold at 4 °C until analysis. PCR amplicons were analyzed on 1.25%

TBE gels stained with ethidium bromide. PCR products were cloned with the Topo TA Cloning Kit into pCR2.1 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Inserts were PCR amplified using primers M13F and M13R. Inserts of the expected size were sequenced at the Life Sciences Core Laboratory Center at Cornell University.

Sequences were aligned first using ClustalW (38). The resulting alignment was then used to create a phylogenetic tree using the MrBayes within Geneious. *Desulfomicrobium thermophilum* was used as an outgroup using HKY85 substitution model (23). Chain length set at 1,100,000 with a subsampling frequency of 200. Priors were set with an unconstrained branch length (23).

### **Quantative PCR.**

Quantative PCR (qPCR) was used to estimate the concentrations of bacterial 16S rRNA gene copies in the enrichment cultures. Reaction mixtures (final volume 25µl) contained 12.5 µL iQ SYBR Green Super Mix (BioRad, Hercules, CA), forward and reverse universal eubacterial primers (31)(200nM each) and 1µl of template DNA. Cycling condition were as follows: 10 minute at 95°C, 35 cycles of 15 seconds at 95°C and 1 minute at 62°C, followed by melting curve analysis from 60 to 95°C to screen from primer dimers using My iQ Single Color Real Time Detection System (Bio-Rad).

Quantification of total bacterial 16S rRNA genes was achieved by analyzing a dilution series of a known quantity of plasmid containing partial 16S rRNA from *Bacillus subtilis* strain 168. DNA concentrations were estimated spectrophotometrically using a ND-1000 spectrophotometer (Nanodrop, Wilmington, DE). Values presented and their standard

deviations are the average of triplicate qPCR reactions from individual enrichment cultures or microcosms.

## Results

### Anaerobic oxidation of ethene in sediment microcosms

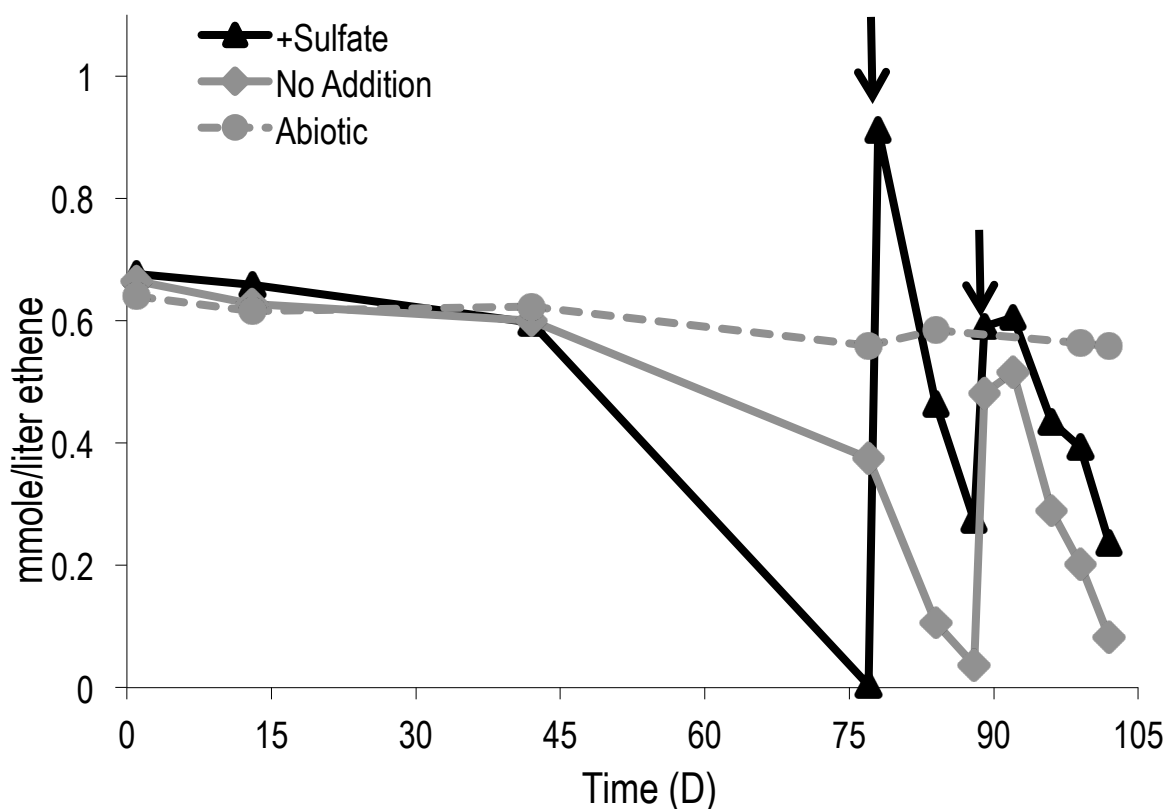


Figure 5.1: Anaerobic oxidation of ethene in microcosms amended with sulfate or unamended in comparison with an uninoculated water blank. Arrows represent an addition of ethene. Representative tubes of triplicates for each treatment are shown. See supplemental Figure 5.1.

Anaerobic microcosms were initially fed ca. 0.6 mmole/liter gaseous ethene. Tubes of equal volume of water and ethene were used as controls for gas loss due to sampling, which failed to show significant decrease in ethene concentration. Figure 5.1 shows a typical microcosm amended with sulfate. Microcosms amended with sulfate, depleted ethene within 74 days, and consumed another dose of ethene after 12 days. In comparison to

the sodium sulfate amended microcosm, consumption of ethene lagged in the unamended microcosms. In the unamended microcosms, the lag time for initial oxidation was quite pronounced, and decreased with further doses of ethene (Figure 5.1) indicative of a biologically mediated reaction. The first dose of ethene was consumed after 88 days with the second dose consumed in another 14 days. Sulfate was the most likely terminal electron acceptor in all these cases since the unamended microcosm only slightly lagged behind the sulfate amended microcosm and endogenous sulfate levels in sediments were elevated (1.8mmol/liter). Microcosms amended with Fe (III) oxide showed the same oxidation rates as in the unamended microcosms. No methane or ethane was detected in these microcosms.

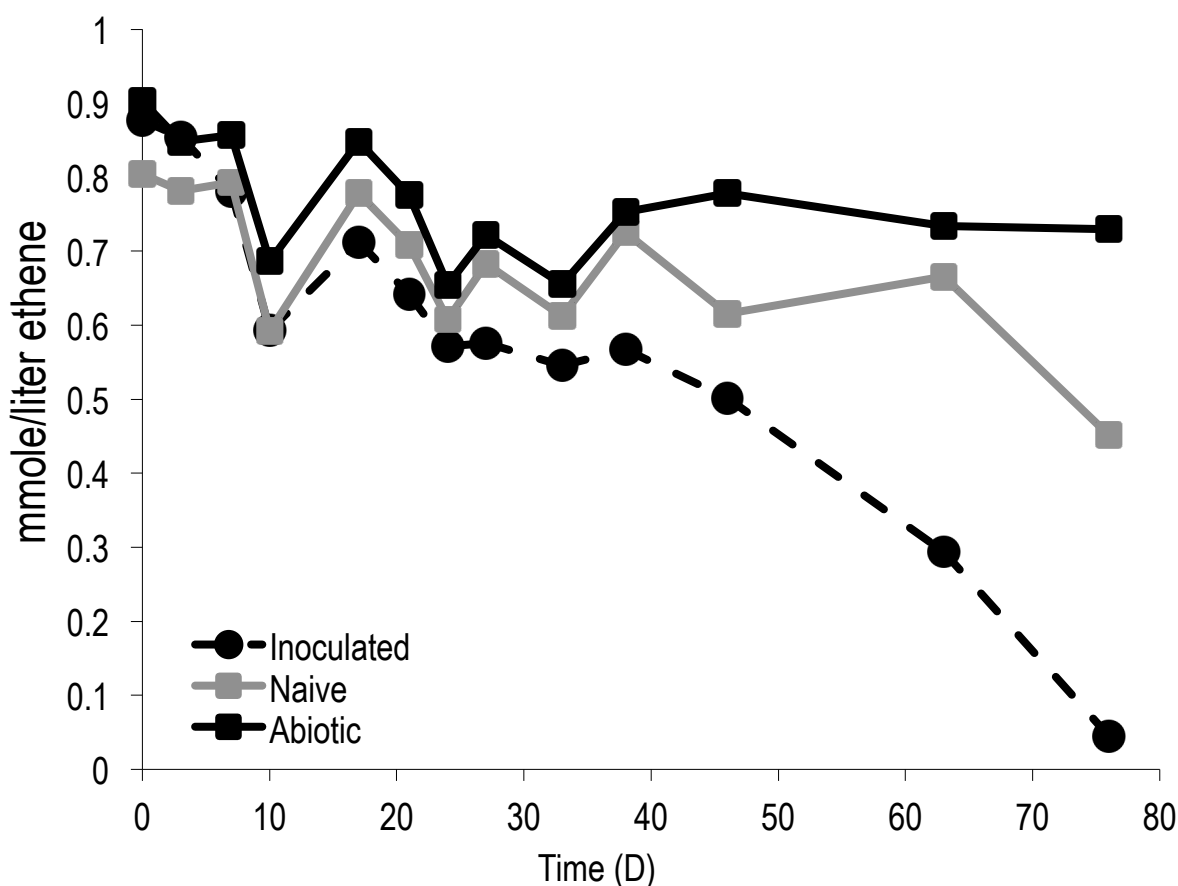


Figure 5.2: Anaerobic oxidation of ethene in naïve and inoculated sediments. Representative tubes of triplicate for each treatment are shown above. All data in supplemental Figure 5.2.



Examination of the effect of addition of sediments enrichments that had consumed doses of ethene on microcosms containing “naïve” sediments showed a decrease in lag time. Naïve sediments were inoculated with a slurry (ca.1%v/v) from a representative vial amended with sulfate that had consumed two doses of ethene. As shown in figure 5.2 the inoculated sediment microcosms consumed ethene faster than the unamended or naïve sediment microcosms. Both of these treatments were amended with additional sulfate as well since this seemed to decrease the lag time as seen with the previous sediment microcosms.

### **Development of ethene oxidizing enrichment cultures**

Sediment slurries from microcosms consuming ethene were transferred into a growth medium based on previous enrichment 1,2-DCB dehalogenating enrichment cultures from the same location (32). Using the optimization parameters from those experiments, ethene-oxidizing enrichment cultures were inoculated into mineral salts medium with amorphous iron sulfide as the reducing agent and a vitamin solution (2, 28). Fermentable substrates such as Casamino Acids or yeast extract were not added to the enrichment cultures so that ethene was the sole electron donor and no other carbon source besides CO<sub>2</sub>.

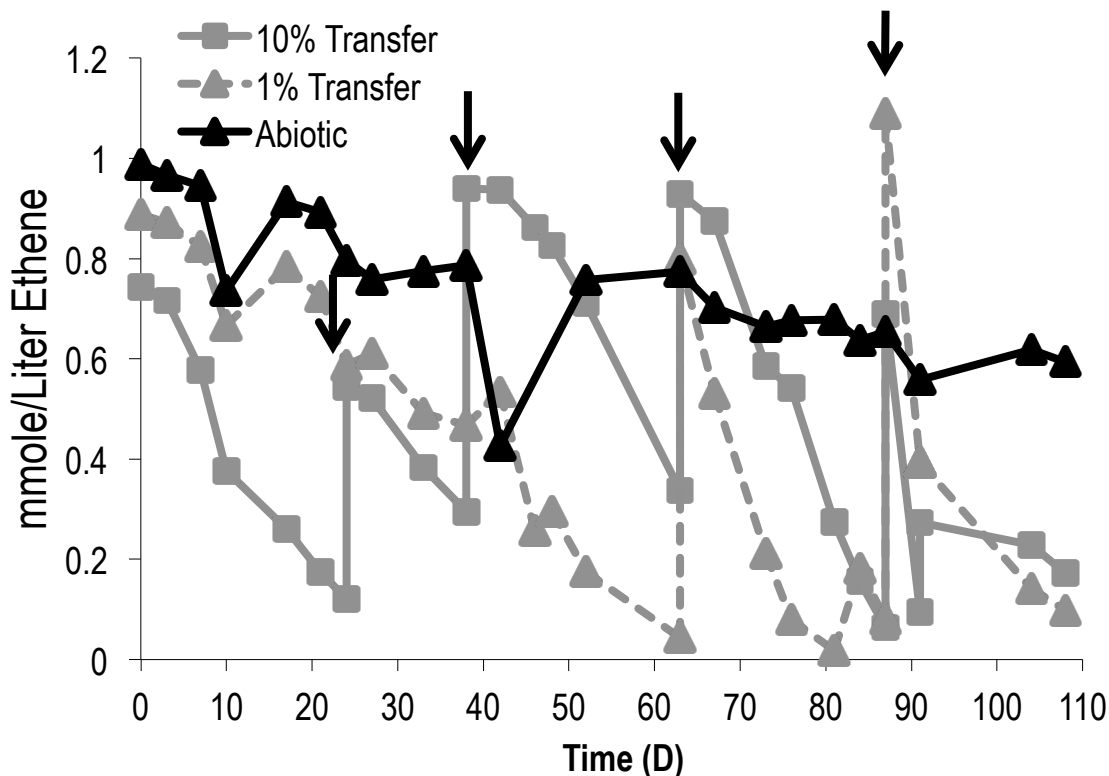


Figure 5.3: First generation enrichment culture inoculated with either a 10% or 1% (vol/vol) sediment slurry from ethene-oxidizing microcosms amended with sulfate. Arrows represent an addition of ethene. One representative tube out of a triplicate set is shown. All data in supplemental Figure 5.3.

First generation transfers to medium containing ethene as the sole organic carbon and electron donor were inoculated with 10% or 1% sediment slurry from a sediment microcosm amended with sulfate that had consumed two doses of ethene. As seen in figure 5.3, the enrichment culture inoculated at 10% consumed its first dose of ethene by 24 days, whereas the 1% lagged, and consumed its first dose by day 63. Upon subsequent addition of ethene, consumption rate increased indicating growth of ethene oxidizing organism. After day 91, the enrichment culture inoculated at 10% seemed to stall indicating that all sulfate had been consumed or sulfide had built up to toxic levels. As seen in equation 2, the oxidation of ethene is favorable under sulfate reducing conditions, with 1.5moles of sulfate consumed for every mole of ethene. In this first generation enrichment cultures, the amount

of ethene consumed is less than that of the supplied sulfate. Since this is only a  $10^{-2}$  dilution from sediments, there are many other organisms and organic material that would have been carried over in the inoculum that could be responsible for the consumption of sulfate and production of sulfide.

Second and third generation transfers were created with the same approach, at a 10% and 1% inoculum, both consuming doses of ethene with the 10% cultures lagging for less time than the 1% inoculum (Supplemental Figures 5.3, 5.4). In the third generation culture inoculated at 10%, the first dose of ethene was consumed in 12 days (Figure 5.4). Uninoculated culture medium failed to decrease the amount of ethene. Third generation cultures were further enriched by addition of increasing amounts of ethene. When challenged with the larger dose of ethene, lag time diminished and consumption continued at an increasing rate.

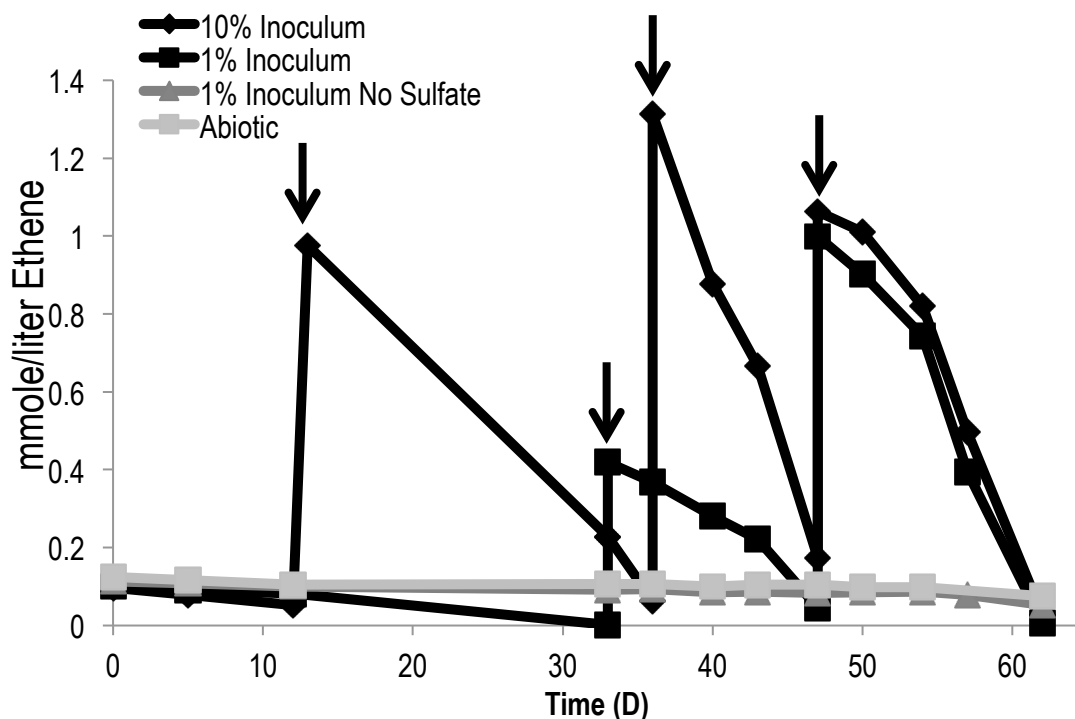


Figure 5.4: Third generation anaerobic ethene-oxidizing enrichment cultures for the oxidation of ethene coupled to the reduction of sulfate in defined medium inoculated at 10% (v/v) and 1% (v/v) with or without sulfate. One representative vial out of a triplicate set is shown. Arrows indicate addition of ethene. Complete data set shown in Supplemental Figure 5.4.

Because enrichment cultures failed to transfer to medium reduced with sodium sulfide, which would have been suitable for following increases in culture optical density, qPCR using “universal” bacterial 16S rRNA gene primers was used to monitor growth in the fourth generation enrichment cultures. These enrichments were inoculated at 2% (v/v). Using this approach it was possible to track an increase of total bacterial 16S rRNA gene copies concurrent with the consumption of ethene. Enrichment cultures fed both ethene and sodium sulfate increased in 16S rRNA gene copy number, whereas cultures lacking either sulfate or ethene failed to increase in bacterial 16S rRNA gene copies to the same level after 64 days. In the culture not amended with sulfate 16S rRNA gene copies per ml increased from  $2.98 \times 10^5$  to  $2.9 \times 10^7$ . The culture not amended with ethene showed a similar

increase in 16 rRNA copy number per ml from  $3.61 \times 10^5$  to  $1.76 \times 10^7$ . However, this is much less than the culture amended with both ethene and sulfate. 16S rRNA gene copies increased from  $2.49 \times 10^5$  to  $1.67 \times 10^8$ . Carryover organic matter or sulfate was present in all the cultures, since cultures without ethene or sulfate were still able to increase in 16S rRNA copy number and produce sulfide.

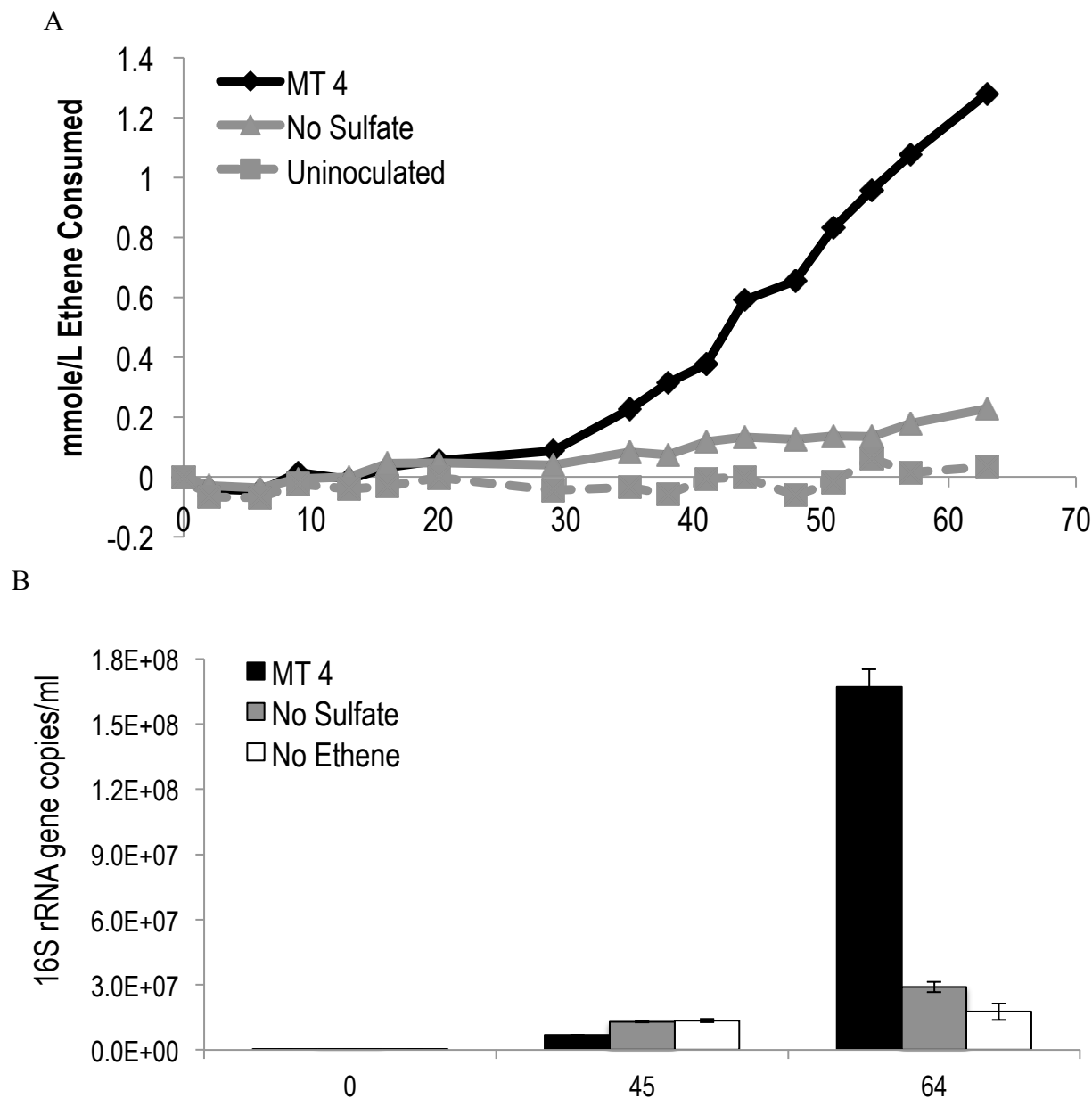


Figure 5.5: Consumption of Ethene (A) linked to increase in 16S rRNA gene copies (B). All Data from the fourth generation (MT4) transfer in comparison to vials without the addition of sulfate and without ethene, and abiotic control. Error bars represent the standard deviation from the mean of triplicate qPCR reactions. Complete data set shown in Supplemental Figures 5.5 and 5.6.

During this growth experiment, sulfide levels were also monitored. In cultures lacking ethene or sulfate, there was little change. However, in cultures amended with ethene and sulfate, sulfide production was evident, with 2.57 mmole/liter sulfide produced

and consumption of 1.27 mmole/liter ethene (Figure 5.5A). According to equation 2, for every mole of ethene consumed, 1.5 moles of sulfide are produced. For this enrichment culture, a ratio of 1.8 sulfide:ethene was measured indicating that other microbes present that are able to produce sulfide from the carryover organic matter. The culture lacking additional sulfate produced 0.3mmole/liter sulfide and consumed 0.2 mmole/liter ethene resulting in a ratio of 1.3 sulfide:ethene. Both values are close to the predicted value of 1.5.

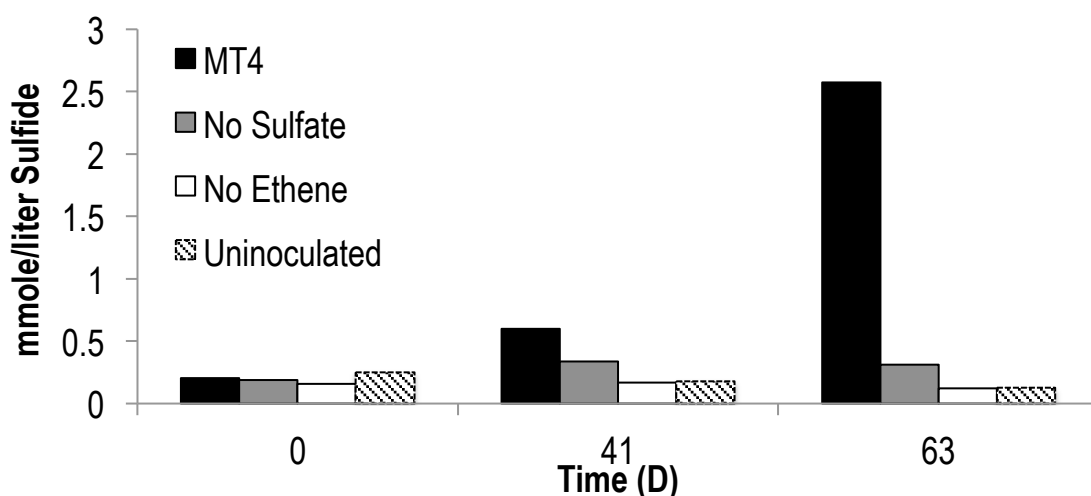


Figure 5.6: Production of sulfide in MT4. All Data from the fourth generation (MT4) transfer in comparison to vials without the addition of sulfate and without ethene, and abiotic control. Time points for sulfide counts correspond to complete consumption of ethene. Complete data set shown in Supplemental Figures 5.5 and 5.6.

### Microbiological characterization

Microscopic observation showed that the dominant morphotype was ovoid cells, 1-2µm in diameter occurring in singlets or doublets. These ovoid cells were most often present within the amorphous iron sulfide used as a reductant for the culture. (Figure 5.7A-C). Two other cell morphologies were often viewed, a small motile curved rod similar to *Desulfovibrio* and the other a slender rod shaped cell, which co-occurred within the iron sulfide particles. The *Desulfovibrio* type cell was often seen outside of the iron sulfide

particles. In successive transfers, the ovoid morphotype became a larger proportion of the total cells.

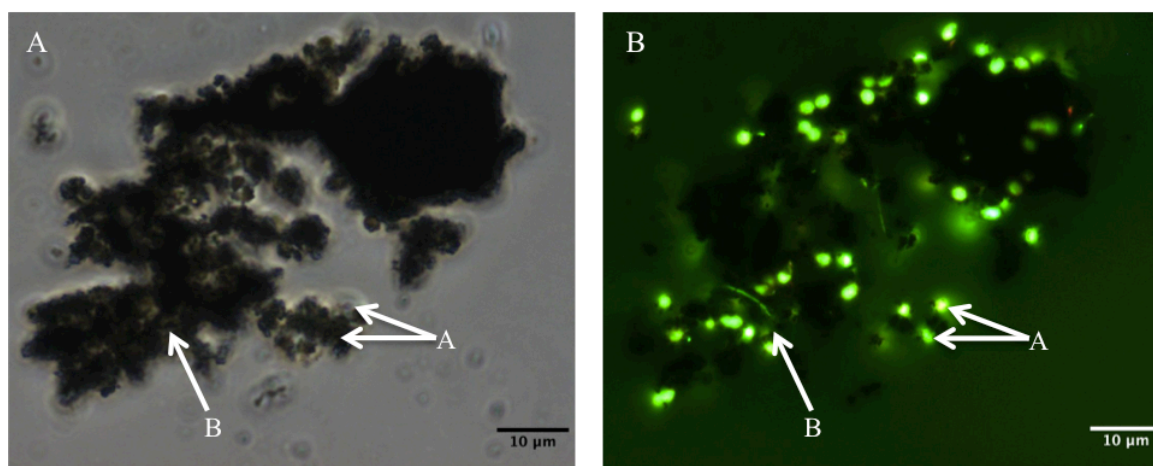


Figure 5.7: Phase contrast (A) and epifluorescence micrograph of a sixth generation culture. The cells were stained with acridine orange and the black particle is amorphous iron sulfide. The ovoid shaped cell type (arrow A) is the dominant member within the iron sulfide particles in comparison to the slender rod shaped cell (arrow B).

A 16S rRNA gene clone library was constructed using universal bacterial primers, from a sixth-generation transfer, which represented a  $10^{-9}$  dilution from the sediments. Amplification with archaeal primers failed to produce a PCR product (data not shown). Of the 22 clones sequenced, 9 distinct sequences were present, and 4 sequences only occurred once. All of the sequences represent phylotypes that are considered anaerobic.

Of the sequenced clones, more than half were most similar by BLAST to *Desulfovirga adipica* strain TsuA1 using the 16S rRNA database for cultured organisms (Table 5.1). Strain TsuA1 was isolated from an anaerobic digester with adipate provided as the electron donor and sulfate, sulfite, thiosulfate or elemental sulfur as electron acceptors (37). Other cultured members of the same clade were members of the genus *Syntrophobacter*, known to couple propionate oxidation to sulfate reduction or syntrophic interactions with methanogens. *Desulfoglaeba alkanexedens*, another relatively close



relative, was isolated for its ability to oxidize n-alkanes coupled to sulfate or thiosulfate reduction (12). This phylotype showed only  $\leq 93\%$  identity with sequences from uncultured microbial communities showing its uniqueness. Similar phylotypes in clone libraries from earlier culture transfers making it unlikely to be a chimera or other artifact.

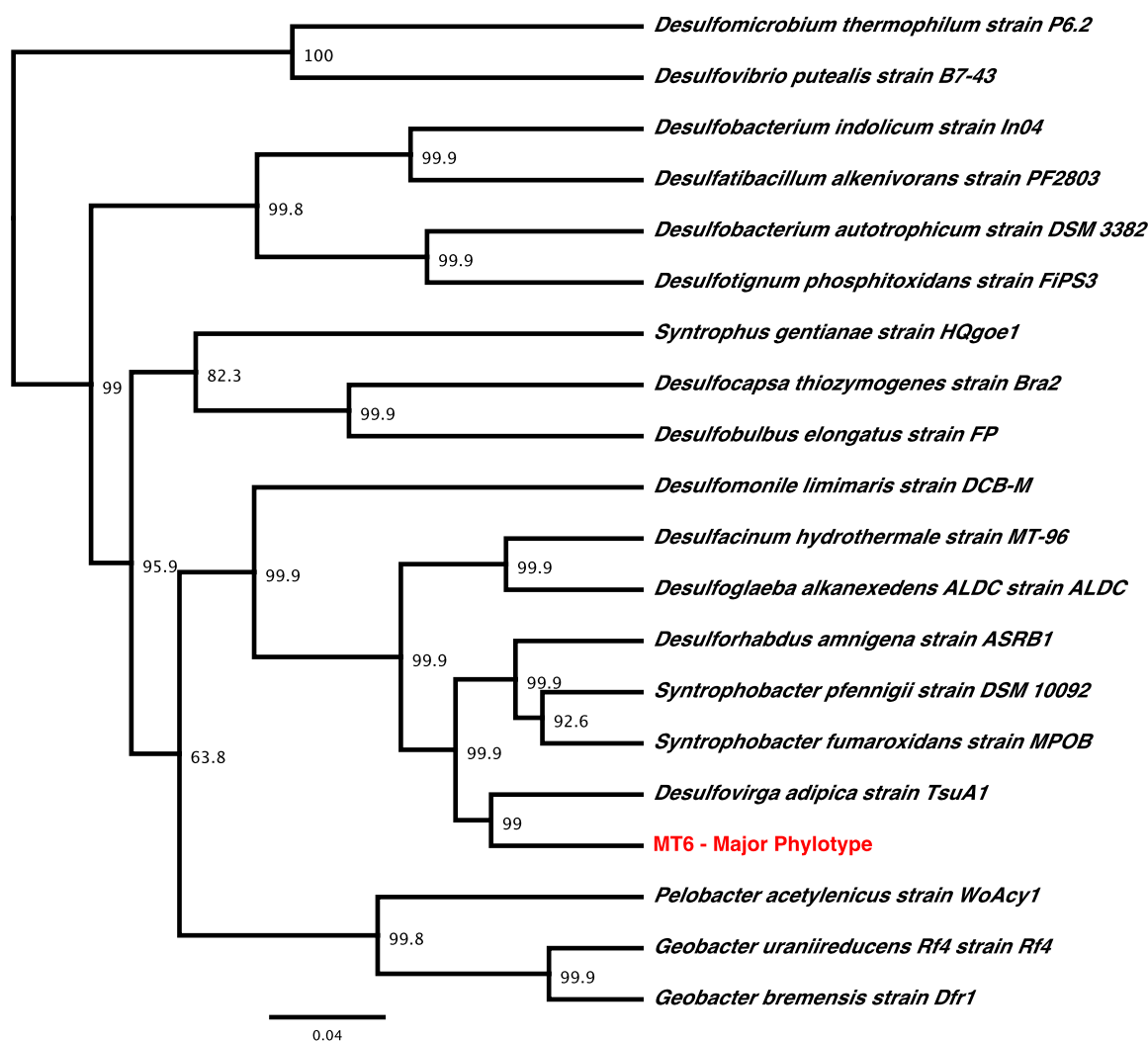


Figure 5.8: 16S rRNA Bayesian gene tree. Sequence in red represents the most numerous phylotype recovered from the clone library. Numbers at node are percent consensus support.

The two next most numerous sequence types were members of the *Spirochaeta*, a phylum not known to anaerobically utilize hydrocarbons, nor were *Levilinea* spp (Tables 5.1 and 5.2). The *Spirochaeta* is closely related to *Treponema* sp. SPIT5, a spirochete

isolated from the hindgut contents of the drywood termite. This strict anaerobe can ferment saccharides with CO<sub>2</sub> and ethanol as the main byproducts. HPLC analysis of the ethene oxidizing culture was unable to detect ethanol or other short chain fatty acids (data not shown). The major phylotype sequence was compared by BLAST to sequences in the nonredundant database, and the top hit was to a uncultured delta proteobacterium sequence from a limonene degrading methanogenic culture.

Table 5.1: Top hits to 16S ribosomal RNA sequences (Bacterial and Archaea) BLAST database and their percentage within library

<b>Best Hit for Sequenced Clone</b>	<b>GenBank Accession Number</b>	<b>Percent Identity</b>	<b>Number of Clones</b>
Desulfovira adipica strain TsuA1	NR_036764.1	90%	59.1%
Treponema sp. SPIT5 strain SPIT5	NR_042486.1	86%	13.6%
Treponema primitia ZAS-2 strain ZAS-2	NR_041714.1	86%	9.1%
Levilinea saccharolytica strain KIBI-1	NR_040972.1	89%	4.5%
Desulfovibrio mexicanus strain Lup1	NR_028776.1	98%	4.5%
Rhodanobacter ginsenosidimutans strain CSC17Ta-90	NR_044467.1	96%	4.5%
Rhodanobacter thiooxydans strain LCS2	NR_041565.1	98%	4.5%

Table 5.1: Top hits to nucleotide collection (nr/nt) BLAST database for sequenced clone types and their percentage within library

<b>Best Hit for Sequenced Clone</b>	<b>GenBank Accession Number</b>	<b>Percent Identity</b>	<b>Number of Clones</b>
UC delta proteobacterium	FN646460.1	93%	59.1%
Levilinea sp. P3M-1	JQ292916.1	97%	4.5%
Bacterial enrichment culture clone	GU080088.1	99%	4.5%
UC Spirochaetaceae bacterium clone B6_81	HQ689205.1	99%	4.5%
UC bacterium clone LHJB-126	JF741946.1	98%	9.1%
UC bacterium clone D12_FB	EU981244.1	99%	4.5%
Bacterium K19(2011)	HQ728406.1	96%	4.5%
UC bacterium SJA-102	AJ009481.1	99%	4.5%
Rhodanobacter sp GR14-4	FJ821729.1	98%	4.5%

## Discussion

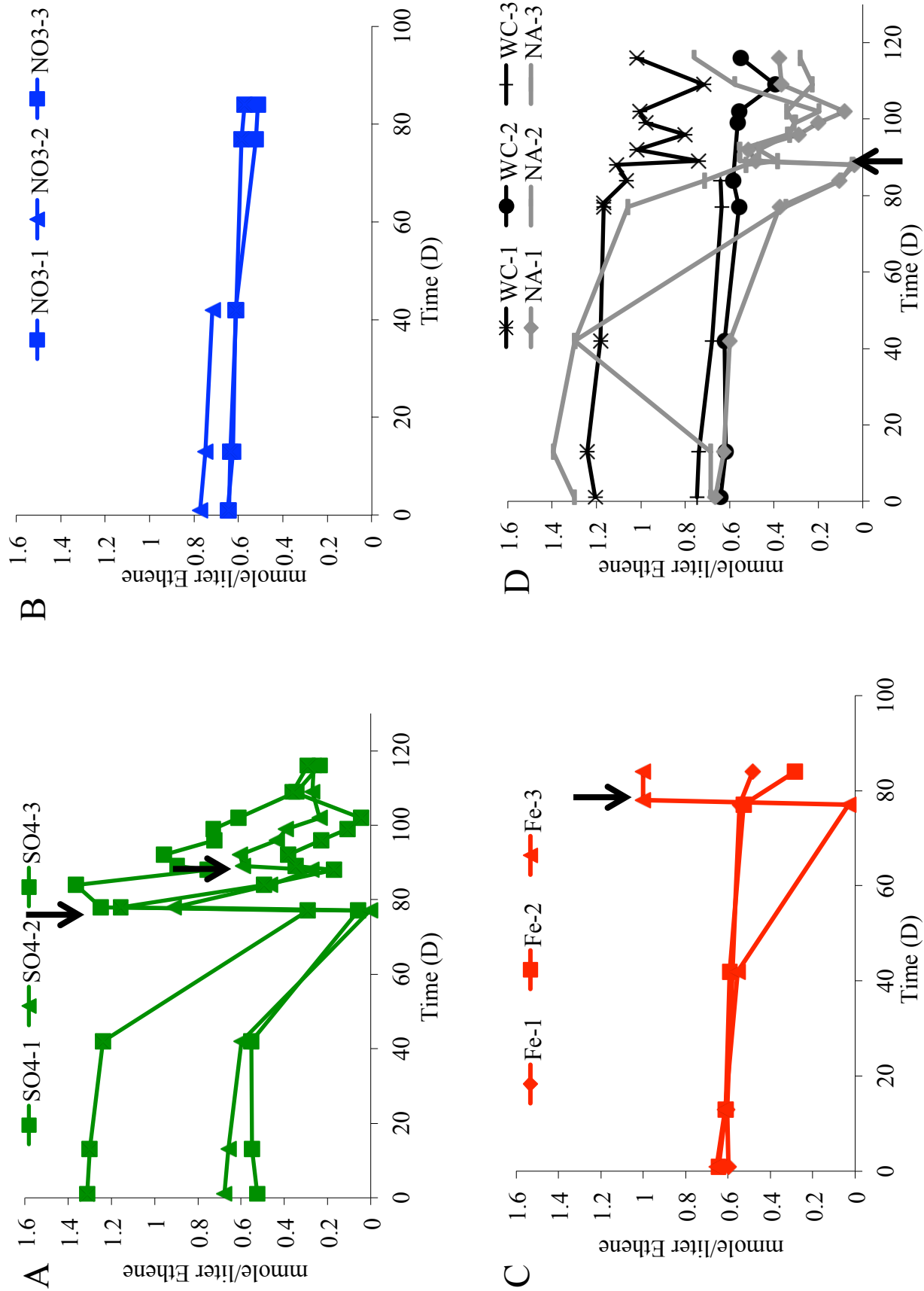
These studies have demonstrated that microcosms and enrichment cultures from Salem Canal sediments can oxidize ethene with the concomitant production of sulfide. Ethene has been considered recalcitrant under anaerobic conditions (35, 36). This study and a previous microcosms study challenge this assumption (7). In this study, the amount of ethene oxidized (4.47 mmole/liter) greatly exceeds that of the previous microcosm study (maximum consumption of 0.075 mmole/liter) (7). Before now, the oxidation of ethene in sediment-free enrichment cultures had not been observed with sulfate.

These sediment-free enrichment cultures could reduce the amount of ethene in the headspaces to below detectable levels without the production of methane or ethane. It is unlikely that methanogens were present since PCR amplification failed to detect any archaeal 16S rRNA sequences (data not shown) and ethene acts as an inhibitor to methanogenesis. Sulfide production and cell growth were observed and were concurrent with ethene consumption. Enrichment cultures were maintained with ethene and CO<sub>2</sub> as the sole carbon sources, and ethene and sulfate as the only redox pair.

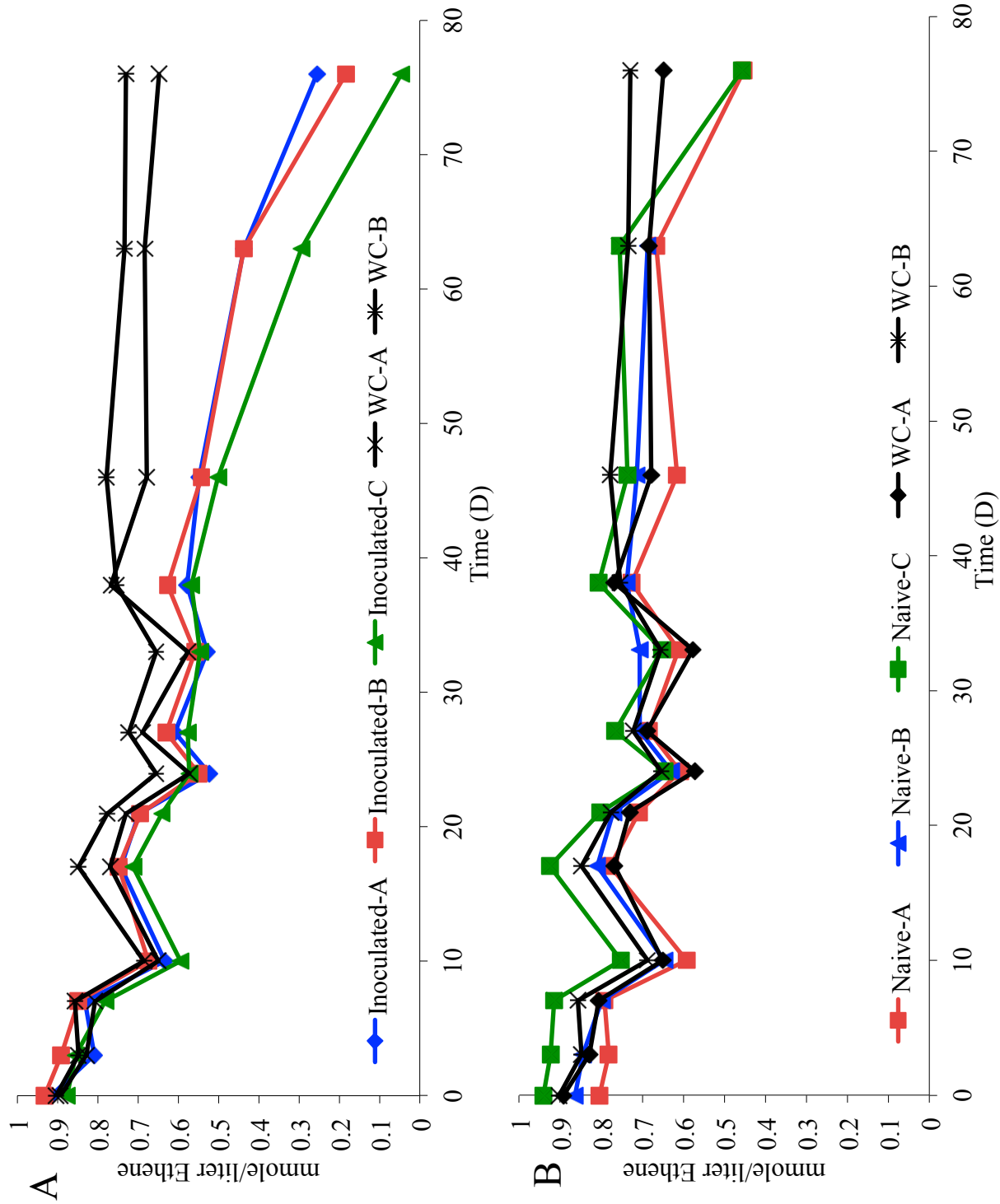
Preliminary data from labeled ethene suggests that ethene is mineralized to CO<sub>2</sub>. Studies are underway to further characterize this culture in respect to substrate utilization. To test electron acceptors, thiosulfate and precipitated sulfur were added in place of sulfate. Thiosulfate and precipitated sulfur are utilized as electron acceptor with ethene as the electron donor, as evident by the loss of ethene over time. The thiosulfate-amended enrichment cultures took 75 days to consume the same dose of ethene that was consumed in 33 days by the sulfate-amended culture. Further testing of S<sup>0</sup> and thiosulfate are underway. Studies of electron donor range is also being tested by serial dilutions with

acetate, propionate, ethanol and ethene will hopefully result in either a pure culture of the ethene oxidizer or give insights to the metabolic pathway that is used in the oxidation of ethene.

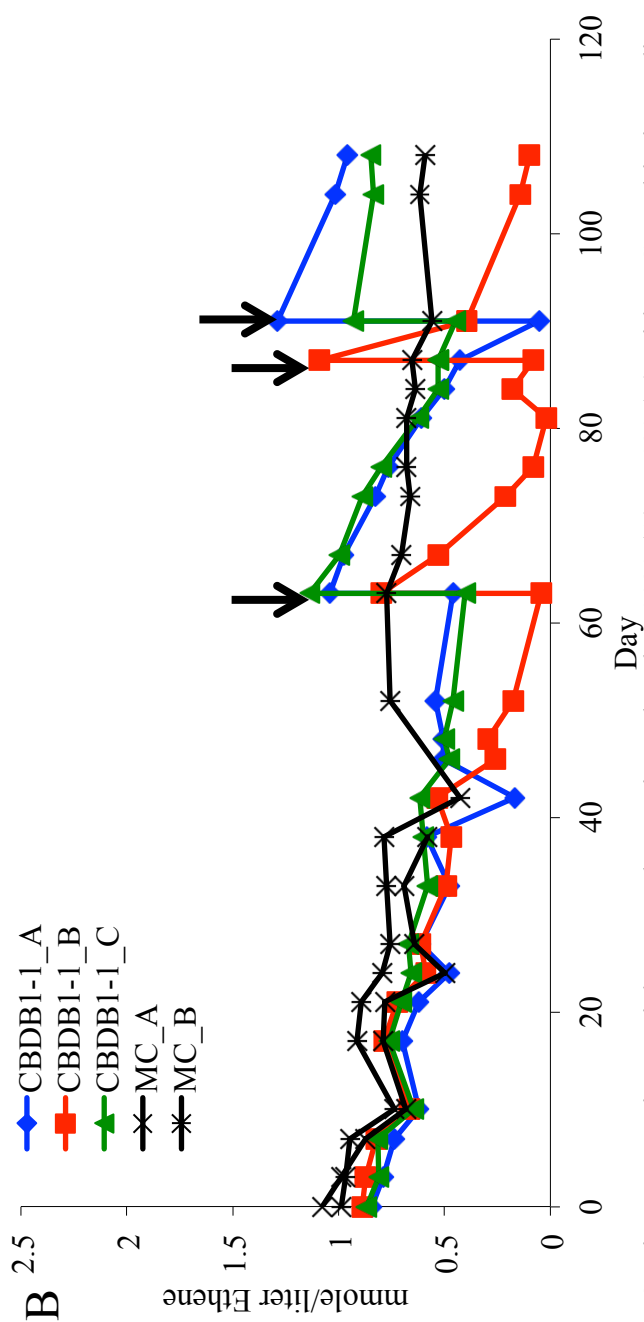
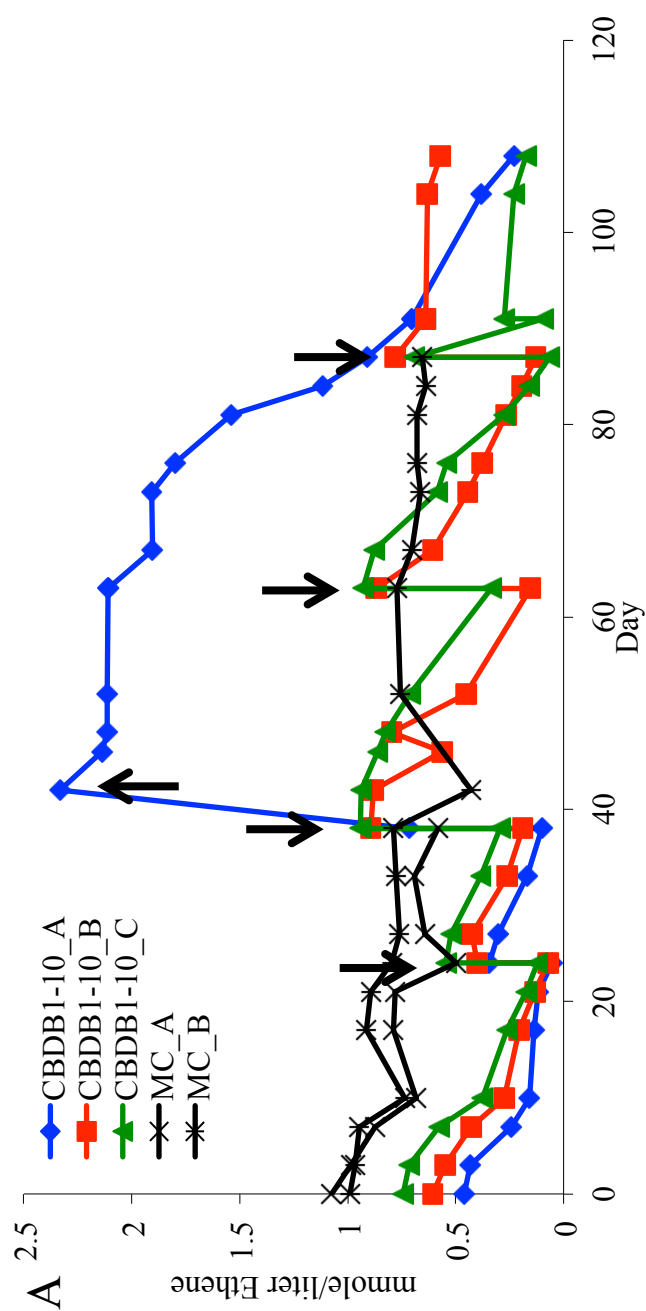
Although ethene is not considered toxic, it is produced as a product of chloroethene dechlorination in anaerobic ground water plumes. The production of ethene is often used in conjunction with the decrease in chloroethene concentration as evidence for bioremediation. This study helps elucidate microbial metabolism of short chain hydrocarbons and can lead to further understanding of metabolite dynamics within contaminated anaerobic plumes, which could be occurring along side reductive dechlorination.



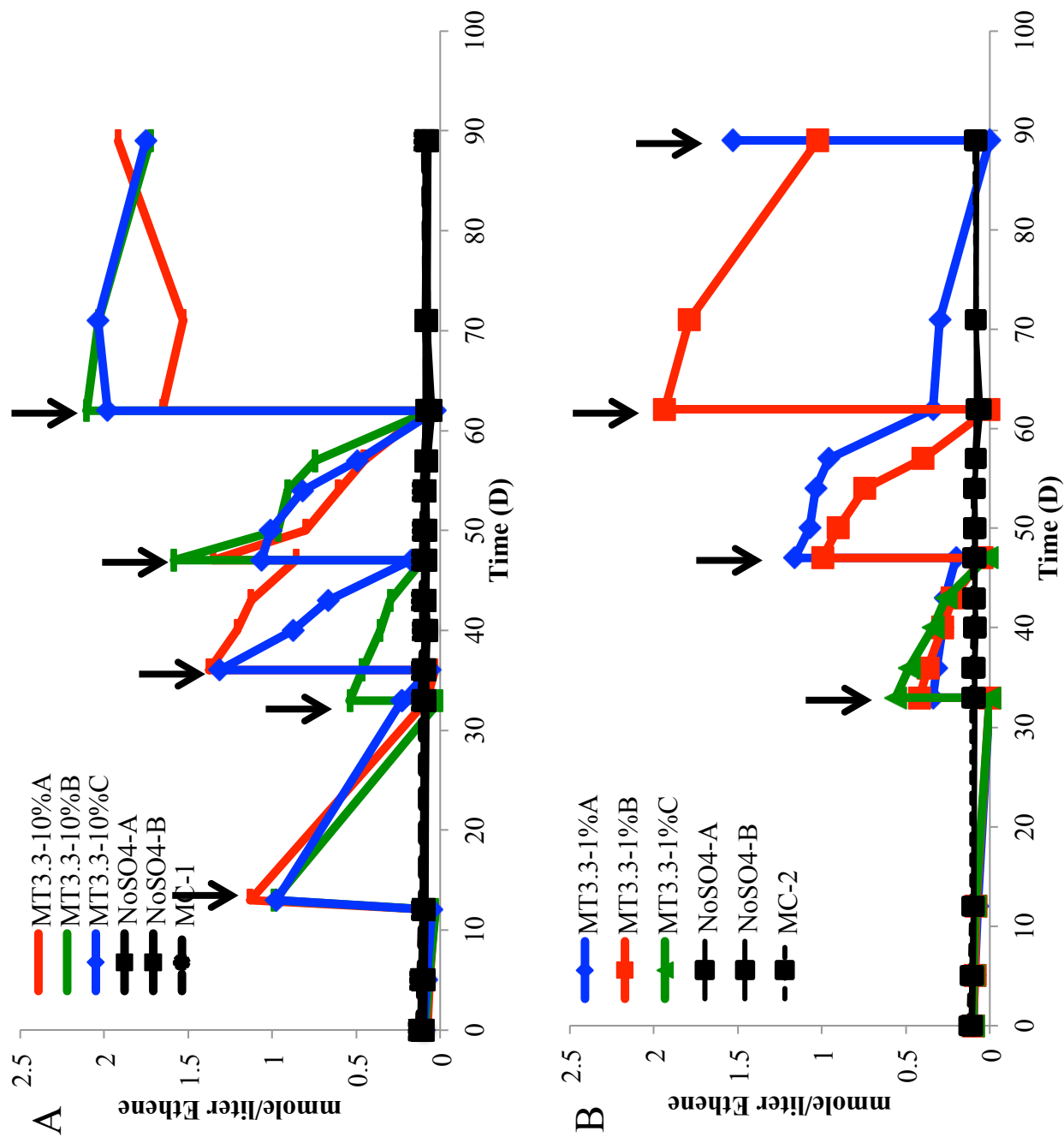
Supplemental Figure 5.1: Salem Canal sediment microcosms amended with either 5mM sodium sulfate (A), 5mM sodium nitrate (B), 5mM amorphous iron oxide (C) or no addition (NA) (D). WC=water control. Arrows represent an addition of ethene.



Supplemental Figure 5.2: Salem Canal sediment microcosms either inoculated (A) from a sediment microcosm at 1% or uninoculated (B). WC=water control.

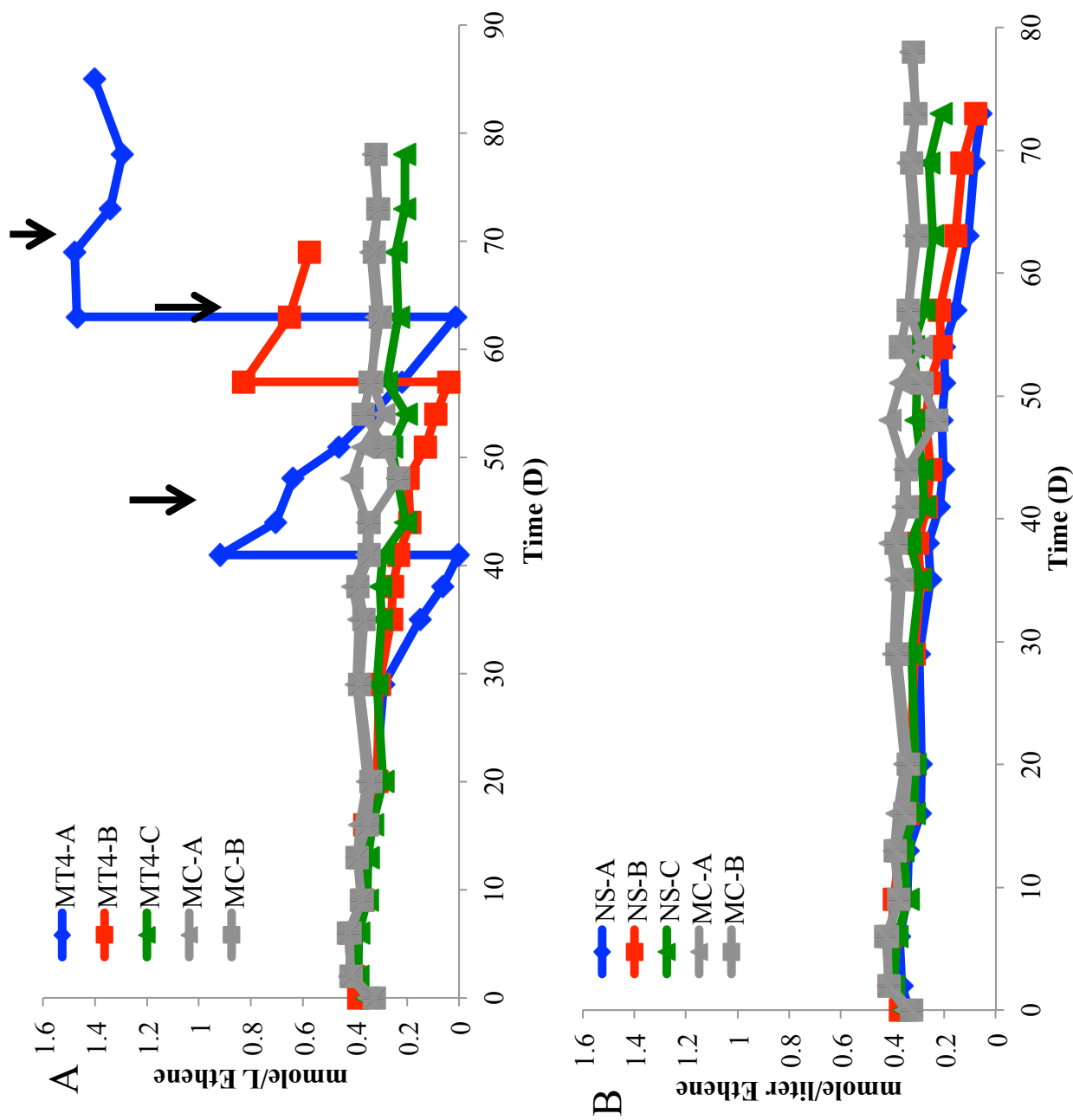


Supplemental Figure 5.3: First generation enrichment cultures, inoculated at 10% (A) or 1% (B) with MC as abiotic medium control. Both treatments inoculated from the same microcosm. Arrows represent an addition of ethene.

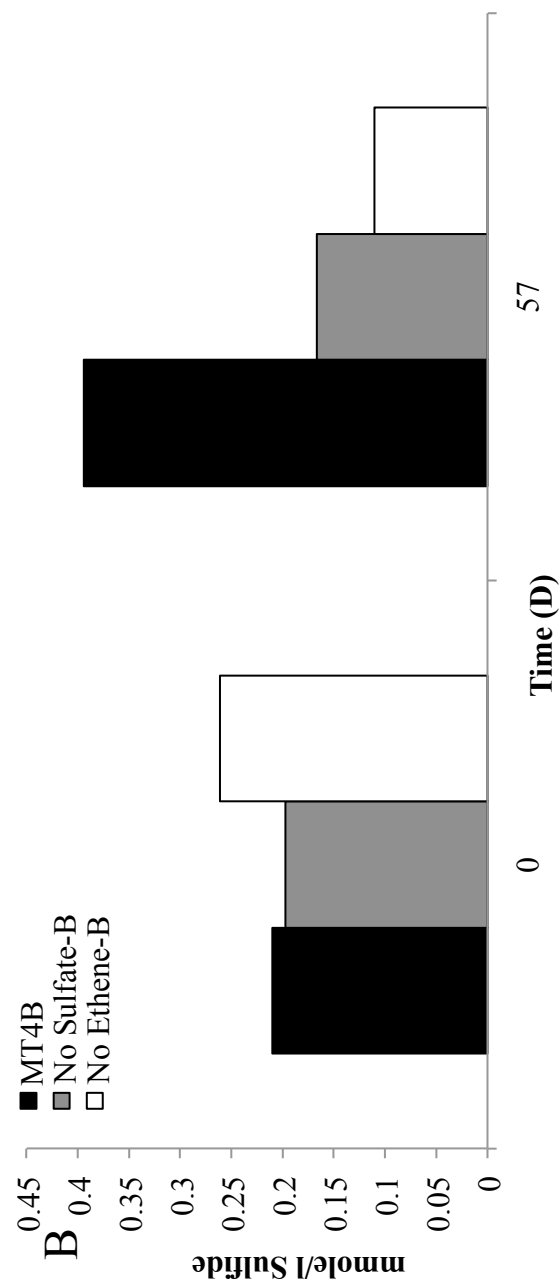
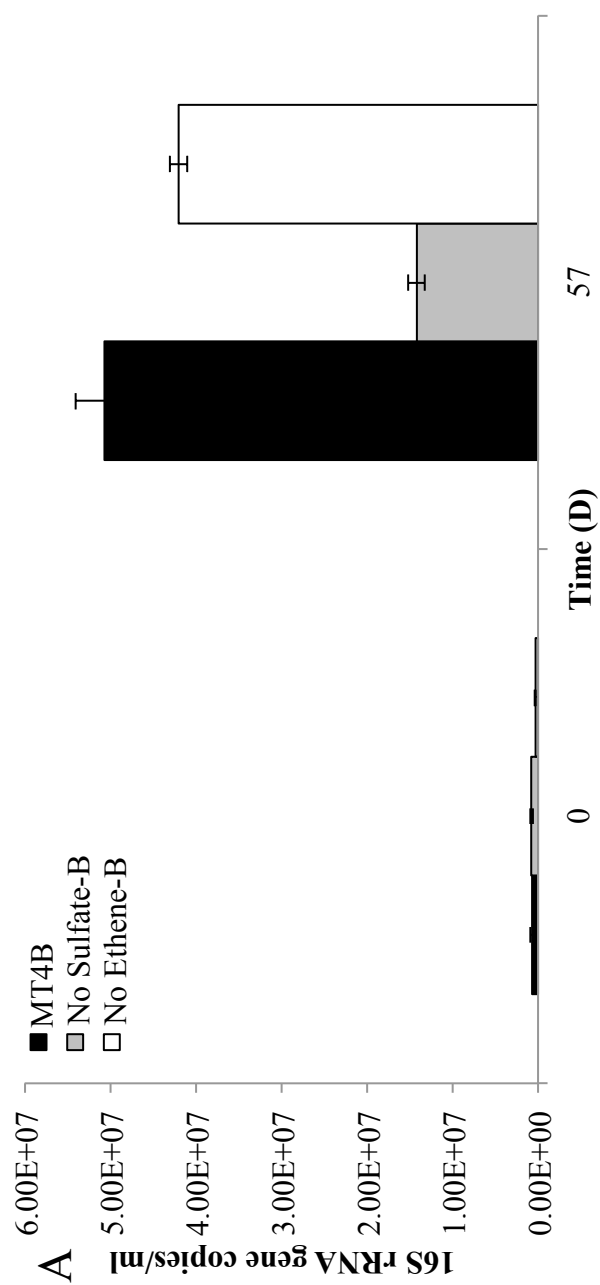


Supplemental Figure 5.4: Third generation enrichment cultures, inoculated at 10% (A) or 1% (B) with MC as abiotic medium control. NoSO<sub>4</sub> was inoculated at 1%. All treatments inoculated from the same second generation enrichment culture. Arrows represent an addition of ethene.

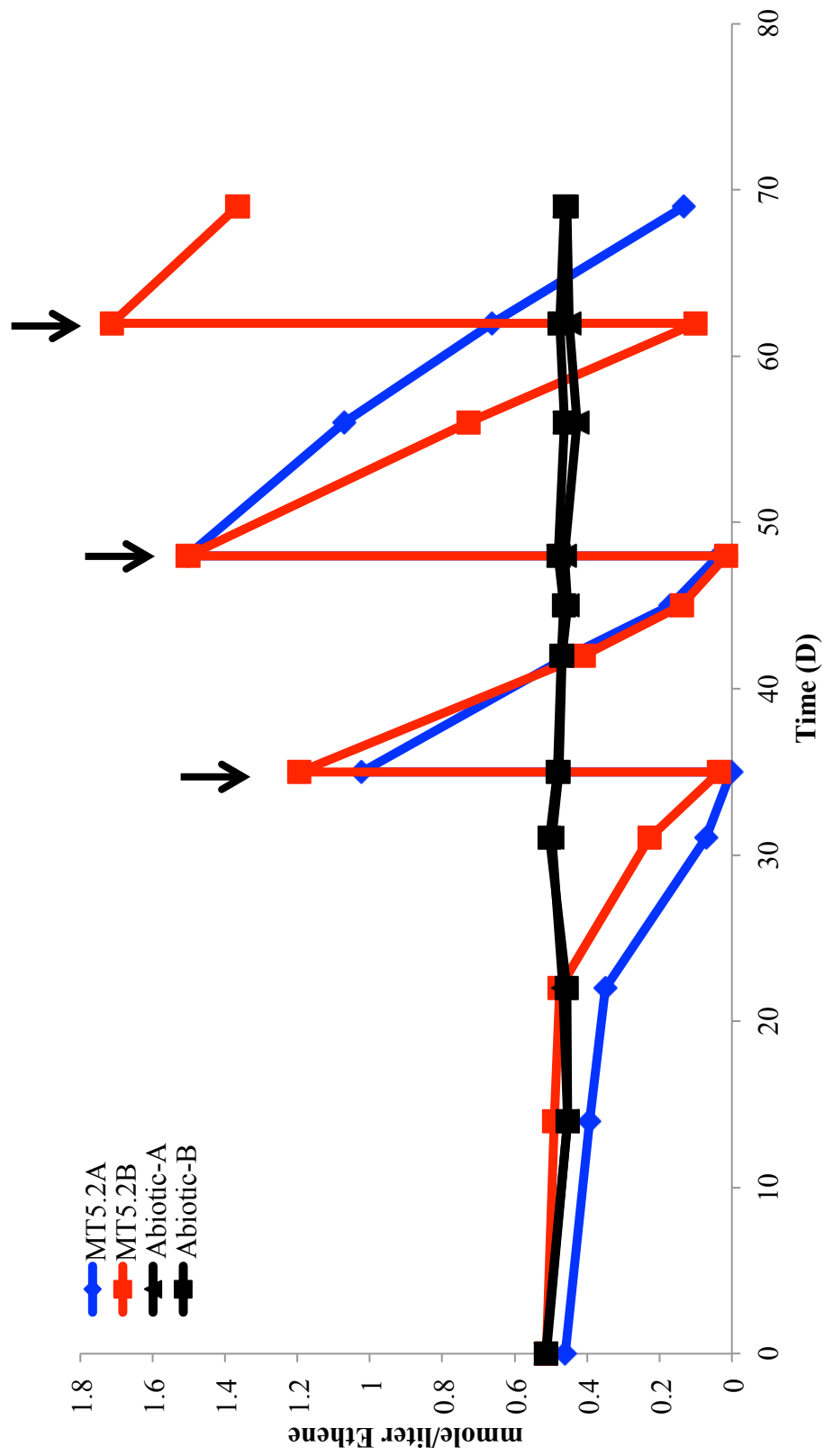




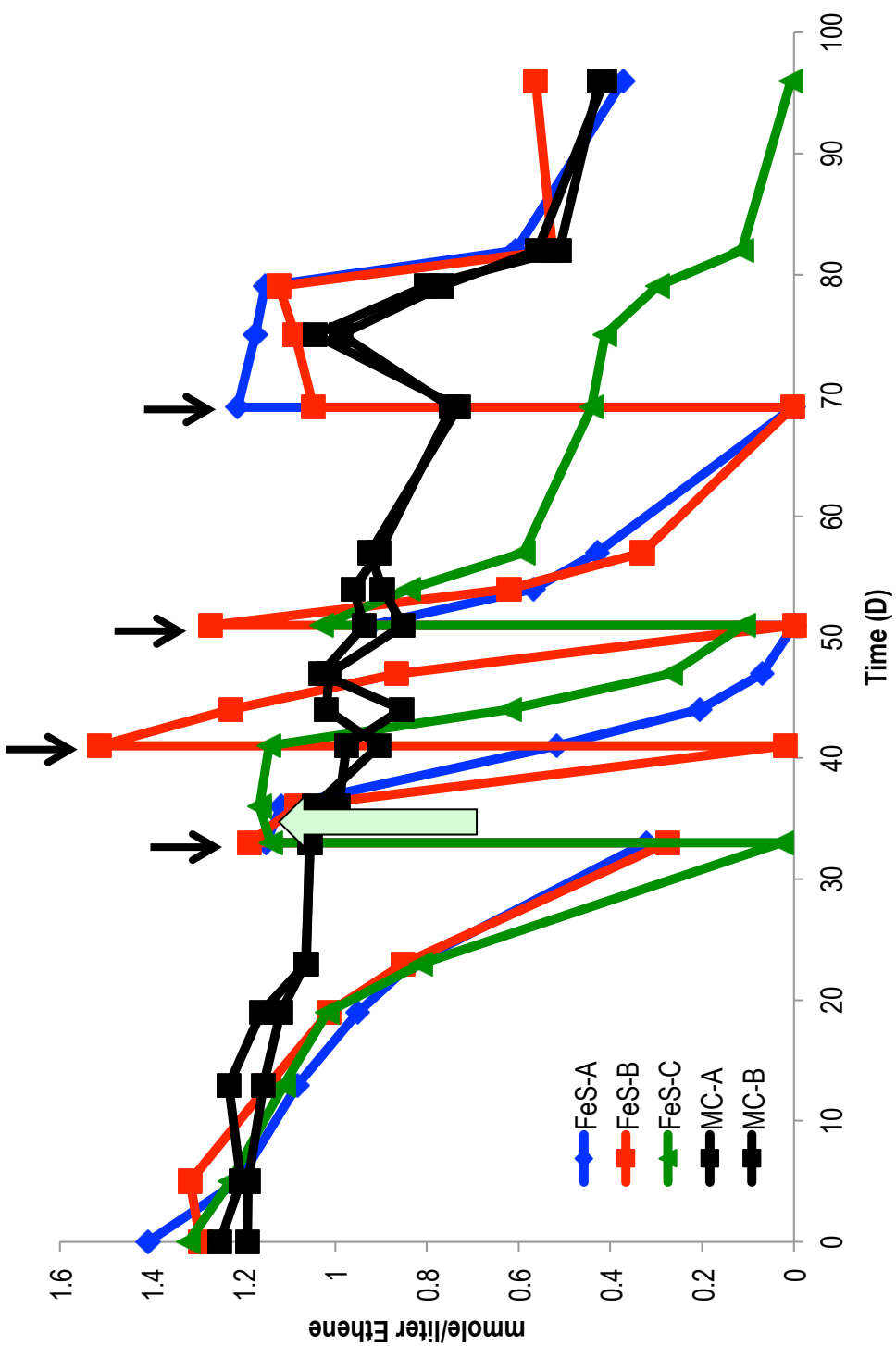
Supplemental Figure 5.5: Fourth generation enrichment cultures, inoculated at 2% with (A) or without sulfate (B) with MC as abiotic medium control. All treatments inoculated from the same third generation enrichment culture. Arrows represent an addition of ethene. After 78 days MT4 failed to show a decrease in ethene concentration, which could be a result of the low inoculum as shown by qPCR on MT4A and MT4B.



Supplemental Figure 5.6: Increase in 16S rRNA gene copies (A) and production of sulfide (B) from fourth generation enrichment culture that consumed the first 0.4 mmole/liter ethene after 57 days. The no sulfate and no ethene controls increased in 16S rRNA copy number but not to the same extent as MT4B. They also showed a slight decrease in sulfide concentration from day zero. The sulfide:ethene ratio for this culture is 1.14. On day 69, this culture was lost (broken).



Supplemental Figure 5.7: Fifth generation enrichment cultures, inoculated at 1%. Both inoculated treatments inoculated from the same fourth generation enrichment culture. Arrows represent an addition of ethene.



Supplemental Figure 5.8: Sixth generation enrichment cultures, inoculated at 1% from the same fifth generation enrichment culture. Black arrows represent an addition of ethene. Green arrow represents when DNA was extracted for clone library analysis.

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## CHAPTER SIX

### SUMMARY, IMPLICATIONS AND FUTURE DIRECTIONS

#### **The Prophage of *Dehalococcoides mccartyi* 195**

*D. mccartyi* strain 195 was the first bacterium isolated that could completely dechlorinate toxic tetrachloroethene to non-toxic ethene (20). In chapter two, the apparent prophage of *D. mccartyi* strain 195 was investigated. A genomic comparison to other sequenced strains of *Dehalococcoides* shows this phage to be unique to strain 195. Related prophages have a genome similar to phages of *Firmicutes*. Those experiments showed the prophage to be active and inducible. This phage appears to have been associated with strain 195 since it was brought into pure culture (19).

Still unknown about this phage is the nature of its genome, whether circular or linear. *Siphoviridae* have a linear chromosome whereas *Fuselloviridae* have a circular chromosome (1, 2). The morphological differences seen between the shape and the predicted shape might indicate that this phage has made many adaptations to life in culture with strain 195. The phage particle exit mode does not appear similar to those of phages within the *Siphoviridae* family. Perhaps this is another adaptation of the phage to long-term survival within strain 195 or the adaption to exit a cell without peptidoglycan.

This phage appears to be active throughout the life cycle of strain 195 as seen by TEM without induction or applying stress to the culture. Phage genomic comparisons between different labs and to the original sequence of strain 195 might show evidence for phage and host coevolution. Data from microarray studies show many of these genes to be active over a range of respiration rates (15). By metagenomics studies, prophages

associated with the *Dehalococcoides* spp. in the ANAS and KB-1 communities have been identified (25). Further study of the *Dehalococcoides* phage dynamics might allow for the development of biomarkers for these interactions.

The presence of a phage within an organism used in bioremediation of contaminated groundwater could impact the ability to achieve complete detoxification of a site. If the phage becomes active during bioremediation, PCE or TCE would fail to be dechlorinated. Monitoring of phage genes or protein presence could be indicative of a failure to bioremediate.

### ***Dehalobacter* Genomes**

In chapter three the analysis of three *Dehalobacter* genomes showed a distinct and diverse clade of reductive dehalogenase enzymes. These bacteria are only able to obtain energy through the reductive dechlorination of chloroorganic compounds such as 1,2-dichlorobenzene (DCB) (21) whereas other strains of *Dehalobacter* have been shown and isolated on chloroform and tetrachloroethene, respectively (11, 14). The genome of the DCB utilizing strains show evidence for specialized growth on chloroorganic compounds. There are no enzymes or metabolic pathways for the utilization of fatty acids or other electron acceptors, which is in contrast to the nearest sequenced phylogenic relative, *Desulfitobacterium hafniense* spp. (16, 22). These organisms, by genomic analysis, would be able to cope with environmental stresses by chemotaxis or forming endospores.

Genomic analysis showed these three strains of DCB dechlorinating *Dehalobacter* to be closely related and to share many metabolic pathways; however their

complement of reductive dehalogenase enzymes show possible metabolic redundancies and overlapping activities between strains. Since the reductive dehalogenases of *Dehalobacter* are divergent from other reductive dehalogenases of known function, further analysis on the specific function of these enzymes will further our understanding of this environmentally important reaction.

### **Vinyl Chloride Oxidation**

Vinyl chloride (VC) is a known human carcinogen and common groundwater contaminant (7). Evidence suggests that anaerobic VC oxidation is possible though studies have yet to link this activity to a specific organism or consortia. Enrichment cultures reduced the concentration of VC without the production of ethene under apparent anaerobic conditions. As shown in chapter four, while investigating the electron acceptor for this activity, only oxygen as electron acceptor showed transferrable activity. This activity was attributed to an organism, a *Mycobacterium* spp., which are known to be able to degrade VC and other anthropogenic compounds (3, 5). This organism was shown to be present within the original enrichment cultures and was most likely responsible for the apparent anaerobic VC oxidation activity in the microcosms.

The inadvertent oxygen contamination allowed for the VC oxidizing mycobacterium to grow at oxygen levels lower than what could accurately be detected (10). This observation could lead to reduce uncertainty at field sites if mycobacteria are detected on the anaerobic edge of a contaminant plume. The strain of mycobacteria isolated in this study is also capable of growing on ethene to the same extent as VC, which is similar to other aerobic VC oxidizing bacteria (4, 5, 8, 12, 17).

## Ethene Oxidation

In chapter five, the anaerobic oxidation of ethene in sediment microcosms and enrichment cultures was examined. The oxidation of ethene was coupled to the reduction of sulfate to sulfide and preliminary studies show ethene to be mineralized to CO<sub>2</sub>. The dominant phylotype present within the anaerobic ethene oxidizing enrichments is closely related to another known sulfate reducer that is able to oxidize adipate, *Desulfovirga adipica* strain TsuA1 (24).

Ethene is not a priority contaminant, however it is produced by the petrochemical industry (13) and through the reductive dechlorination of PCE (9), can have negative impacts on fruit production (23). Bacteria have been characterized with their biochemical pathways deciphered for the aerobic oxidation of ethene (4, 6, 8, 18), though this is the first report of anaerobic oxidation of ethene at greater than tracer quantities.

Continued work should focus on isolation and characterization this organism's substrate range. Studies are underway to further characterize this microbe's metabolic capabilities and substrate ranges. Of the substrates tested, ethene concentrations are reduced in the presence of S<sup>0</sup> and thiosulfate, similar to *D. adipica* TsuA1. The metabolism of fatty acids by this enrichment culture in the presence of sulfate is an on-going study within the laboratory.

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